MOLECULAR AND METABOLIC BASES OF TETRAHYDROBIOPTERIN-RESPONSIVE PHENYLALANINE HYDROXYLASE DEFICIENCY

Dissertation

zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich
von

Caroline Heintz
aus Luxemburg

Promotionskomitee
Prof. Dr. François Verrey (Vorsitz)
Prof. Dr. Nenad Blau (Leitung der Dissertation)
Prof. Dr. Beat Thöny
Prof. Dr. Marc Donath

Zürich, 2012

Abstract

Phenylketonuria (PKU) is the most common inborn error of human amino acid metabolism. The disorder is characterized by elevated levels of phenylalanine (Phe) in the blood, due to a deficiency in phenylalanine hydroxylase (PAH), the hepatic enzyme oxidizing Phe to tyrosine (Tyr) in the presence of O_2 and the cofactor tetrahydrobiopterin (BH_4). The accumulated Phe is neurotoxic, leading to CNS Tyr deficiency and disturbed neurotransmitter synthesis. If not treated early in life by dietary restriction of essential Phe, the metabolic phenotype is severe resulting in disturbances in brain development and cognition.

PKU is inherited in an autosomal recessive manner with a broad mutational spectrum leading to heterogeneity among PKU patients. Many patients are compound heterozygous with the genotype being the main determinant for the phenotype and not the single allele alone. The variation of dietary protein combined with genetic alteration lead to a multifactorial cause for hyperphenylalaninemia. However, there are many unsolved aspects between the patient's genotype, its predicted effect on enzyme function, and the associated metabolic phenotype.

Oral supplementation of the PAH natural cofactor BH_4 is the first pharmacological therapy for patients with PKU, but there is still limited information on the molecular basis of BH_4 responsiveness. About 20 - 30% of PKU patients are known to respond to BH_4 administration by a significant reduction of their blood phenylalanine levels, i.e. they can be treated with BH_4 instead of a low Phe diet or in combination with the diet.

In this work, we aimed at gaining further insight into the metabolic and molecular mechanisms of determining BH_4 responsiveness. The thesis project outlines a molecular, *in vitro* analysis of several common, and also new PKU mutations and testing of their response to BH_4 in a mammalian cell system.

In order to gather more genotype information and select mutation combinations for analysis, two patient studies are described. They include the large-scale genotyping of PKU patients previously tested for BH_4 responsiveness. Phe levels as well as loading test data were available from most of the 750 patients from Turkey and Croatia. The large dataset permitted the division into several smaller groups to discriminate between distinct BH_4 loading test protocols and BH_4 formulation used.

Based on these data we calculated correlations of the genotypes with response to BH_4, phenotype, and with *in vitro* residual PAH activities. The correlations showed a relative high

residual PAH activity is required for response to BH_4 supplementation. Among the Turkish PKU patients, a relatively high proportion (22%) of potential candidates for the BH_4 therapy was detected. Furthermore, the studies highlighted that single allele mutations are not reliable for the selection of potential PKU candidates for pharmacological therapy with BH_4. Considering both studies, twenty-four novel mutations were detected. The assembly of more than 700 genotypes, phenotypes and BH_4 loading tests data further extended the BIOPKU database. The database is public and new patients with documented non-responsive genotype may not need to undergo BH_4 testing.

The quantification of PAH activity expressed in cultured cells or in animal tissues is performed by a novel tandem mass spectrometry assay. Mass spectrometry allows the use of stable isotopes for Phe and Tyr quantification and PAH activity measurement. Among the discordances reported with effective prediction of a mutation's effect on enzyme function and residual PAH activity, is the use of various assay protocols and quantification methods. Therefore, a validated, reproducible, and more specific method compared to previously used approaches for determining *in vitro* PAH activity was established. LC-MSMS is a powerful technique where low detection limits were found for both amino acids and thus it requires lower sample amounts for accurate determination of PAH activity.

Furthermore, several particular mutations found in Turkish PKU patients were selected for detailed molecular analysis. The study included investigating the association of an intronic mutation in *PAH* intron 10 with BH_4 responsiveness and two other mutations in *PAH* exon 11. Discordant PAH activity results were found for these mutations, as well as their location in important splicing regulatory elements pointed to an implication on *PAH* exon 11 mRNA processing. *PAH* minigene constructs and EBV-transformed PKU patient cell lines harboring these mutations were established and analyzed. More than one transcript was detected upon amplification of exons 10 to 12 from the minigenes and the patient cells carrying the mutations of interest. Sequencing confirmed skipping of *PAH* exon 11 during mRNA processing. Affinity purifications showed abolished binding to specific SR proteins of RNA oligonucleotides, carrying the two missense mutations investigated. *PAH* exon 11 is vulnerable and recognition of exon 11 is subtle with mutations in splicing regulatory sites.

Only the combination of the two mutant PAH alleles that determines residual PAH activity *in vivo*, and the individual mutations of a patient should not be viewed by themselves. The final section of this thesis presents the setup and validation of a mammalian cell test system with engineered plasmid vectors. The system allows transient co-expression of wild type or mutant PAH variants of previously selected genotypes and measurement of

PAH activity by LC-MSMS and expression levels, in presence or absence of exogenous BH_4. The selection of genotype combinations was done referring to the previously mentioned patient studies and the BIOPKU database. The co-expression of two distinct PAH mutant alleles revealed possible dominance effects (positive or negative) by one of the mutations on residual activity as result of interallelic complementation. Treatment of the transfected cells with sepiapterin as BH_4 precursor showed an increase in residual PAH activity with several mutations co-expressed.

In summary, the results presented herein provide additional information and elucidation on PKU genotypes, phenotypes, and response to BH_4 as a reference available for clinicians, health care professionals, and researchers for diagnosis and establishment of tailored treatment of patients.

Zusammenfassung

Die Phenylketonurie (PKU) gehört zu den häufigsten, angeborenen Störungen im menschlichen Stoffwechsel der Aminosäuren. Durch einen Defekt im Leberenzym Phenylalanin Hydroxylase (PAH) wird die essentielle Aminosäure Phenylalanin (Phe) unvollständig zu Tyrosin (Tyr) umgewandelt. Dieser Vorgang führt, unter der Beteiligung von Tetrahydrobiopterin (BH_4, Kofaktor) und O_2, zur Anreicherung von Phe im Blut. Daraus resultieren Störungen im Tyrosinstoffwechsel sowie auch in der anschließenden Neurotransmittersynthese. Bei strenger, von Anfang an eingehaltener Phe-reduzierter Diät können die neurotoxischen Schäden von Phe und der Mangel an Folgemetaboliten in der Hirn- und Wahrnehmungsentwicklung vermindert werden.

Die Vererbung von PKU erfolgt autosomal rezessiv mit einem breiten Mutationsspektrum und führt zu einer Vielfalt an metabolischen Phänotypen. Die meisten Patienten haben einen gemischt heterozygoten Genotyp, welcher für die Ausprägung des Phänotyps im Gegensatz zum einzelnen mutierten Allel bestimmend ist. Die Schwankungen in der Nahrungsproteinaufnahme in Kombination mit der Genänderung sind multifaktorielle Ursachen für Hyperphenylalaninämie. Diese Faktoren führen zu verschiedenen Unstimmigkeiten in der Vorhersage des assoziierten metabolischen Phänotyps, aufgrund des Einflusses der Mutationen und deren vorhergesagten Effekts auf die Enzymfunktion.

Die erste pharmakologische Behandlung für PKU Patienten besteht in der Einnahme von BH_4, dem natürlichen Kofaktors von PAH. Es gibt allerdings nur wenig Information über die molekularbiologischen Hintergründe bezüglich des Ansprechverhaltens auf BH_4. Etwa 20 bis 30% der Patienten reagieren auf diese Einnahme durch eine Abnahme der Blutphenylalaninkonzentration und können somit mit BH_4 anstelle der niedrigen Phe-Diät behandelt werden.

Das Ziel der vorliegenden Arbeit ist die weitere Aufklärung der metabolischen und molekularbiologischen Mechanismen der BH_4-Sensitivität. Das Projekt behandelt die molekulare, *in vitro* Untersuchung von mehreren bekannten PKU Mutationen, sowie auch von neuen Varianten. Außerdem wurden die Mutationen auf ihre Sensitivität gegenüber BH_4 in einem Zellkultursystem getestet.

Zusätzliche Genotypdaten und Allelkombinationen für eine detaillierte Studie wurden in zwei Patientenstudien gesammelt. Hierbei wurden 750 PKU-Patienten aus der Türkei und Kroatien genotypisiert, die vorher auf ihre BH_4-Sensitivität getestet wurden. Phe-Werte sowie auch BH_4-Belastungstestdaten wurden miteinander korreliert. Die große Datenmenge

erlaubt die Einteilung in kleinere Gruppen, um zwischen unterschiedlichen Belastungstestvorgängen und BH_4-Formulierungen zu unterscheiden. Zusätzlich wurden die Genotypen mit dem Phänotyp, der BH_4-Sensitivität und *in vitro* PAH-Restaktivitäten korreliert. Die Auswertung zeigte, dass erhöhte Restaktivität notwendig für das Ansprechen auf BH_4 ist. Bei den türkischen Patienten wurden mit 22% relativ viele mögliche Kandidaten für die Therapie mit dem Kofaktor gefunden. Zusätzlich zeigten diese beiden Patientenstudien, dass einzelne Allelvariationen nicht ausreichend sind um zuverlässig Patienten für diese Therapie auszuwählen. Weiterhin wurden 24 neue PAH-Mutationen gefunden. Die Zusammenstellung von den Genotypen, Phänotypen und BH_4-Belastungstestdaten wurde in die öffentliche BIOPKU Datenbank eingetragen. Patienten mit dokumentiertem, nicht-BH_4-sensitiven Genotyp müssen keinem Belastungstest mehr unterzogen werden.

Die Quantifizierung von PAH-Aktivitäten in Zellkulturen oder Tiergewebeproben wurde mit einer neuen Tandem-Massenspektrometriemethode durchgeführt. In der Massenspektrometrie (MS) können stabile Isotope von Phe und Tyr zur Quantifizierung und somit für PAH-Aktivitätsmessungen eingesetzt werden. Verschiedene PAH-Aktivitätsmessungsprotokolle und Quantifizierungsmethoden führen immer wieder zu unterschiedlichen Ergebnissen bezüglich Mutationen und deren Effekt auf die Enzymfunktion und Restaktivität. Aufgrund dessen haben wir eine auf LC-MSMS-basierte, validierte, reproduzierbare und im Vergleich zu anderen gängigen Messmethoden spezifischere Methode zur *in vitro* Bestimmung von PAH-Aktivitäten entwickelt. Diese neue Methode benötigt kleinere Probenmengen und zeichnet sich durch niedrige Detektionsgrenzen für beide Aminosäuren aus.

Eine detaillierte, molekularbiologische Analyse wurde an drei ausgewählten Mutationen aus der türkischen Patientenstudie durchgeführt. Hierbei handelt es sich um eine intronische *PAH* Mutation (Intron 10) mit BH_4-Sensitivität, sowie zwei Punktmutationen im Exon 11 die zum Austausch von Aminosäuren führen. Für diese Mutationen fanden wir widersprüchliche PAH-Aktivitäten. Zusätzlich deutete die Lage dieser beiden Mutationen in Elementen der Spleissregulierung auf einen möglichen Einfluss auf die Prozessierung der *PAH* mRNA. *PAH*-Minigene und EBV-transformierte Zelllinien von PKU Patienten mit diesen Mutationen wurden hergestellt und untersucht. Die Analyse der cDNA Sequenz ergab zwei unterschiedlich lange Transkripte nach der PCR-Amplifikation von *PAH* Exon 10 bis 12. Die Sequenzierung bestätigte, dass es sich hierbei um das Fehlen von Exon 11 handelte. Weiterhin zeigte die Affinitätschromatographie von RNA Oligonukleotiden (tragen die

untersuchten Mutationen in der Sequenz) eine fehlende Bindung zu spezifischen SR Proteinen. Zusammenfassend ist *PAH* Exon 11 labil und Mutationen in spleissregulierenden Elementen führen leicht zu einer verminderten oder ausbleibenden Exonidentifizierung.

Das Zusammenwirken zweier, mutierter *PAH*-Allele bestimmt die PAH-Restaktivität *in vivo*. Daher sollten auch *in vitro* die Mutationen nicht nur einzeln untersucht werden. Im letzten Teil dieser Arbeit wird der Aufbau und die Validierung eines Zellkulturtestsystems mit gentechnisch veränderten Plasmidvektoren beschrieben. Das System ermöglicht die transiente Co-Expression von Wildtyp oder mutierten PAH-Varianten aus ausgewählten Allelkombinationen, sowie die Bestimmung der PAH-Aktivität mittels LC-MSMS und die Menge der PAH-Expression, in Gegenwart oder Abwesenheit von exogenem BH_4. Die Auswahl der Genotypen basierte auf den Daten der Patientenstudien und der BIOPKU Datenbank. Die Co-Expression von zwei unterschiedlichen PAH-Allelen wies mögliche dominierenden Effekte (positive oder negative) durch eine der beiden Mutationen auf die PAH-Restaktivität aufgrund der interallelischen Komplementierung auf. Die Behandlung von transfektierten Zellen mit sepiapterin als BH_4-Vorstufe zeigte einen Anstieg der Restaktivität bei einigen der untersuchten Mutationen.

Zusammenfassend bieten die hier gezeigten Studien zusätzliche Informationen und Aufklärung bezüglich PKU Genotypen, Phänotypen und BH_4-Sensitivität. Diese Arbeit dient als Referenz für Mediziner, Therapeuten und Forscher zur Diagnose und zur Erstellung einer auf den Patienten zugeschnittenen Behandlung.

Contents

Contributions to the publications

I) Molecular Genetics and Impact of *In Vitro* Residual Phenylalanine Hydroxylase Activity on Tetrahydrobiopterin Responsiveness in Turkish PKU Population

Steven F. Dobrowolski[1], Caroline Heintz[1], Trent Miller, Clinton Ellingson, Clifford Ellingson, Işıl Özer, Gulden Gökçay, Tolunay Baykal, Beat Thöny, Mübeccel Demirkol, Nenad Blau

[1]The first two authors should be regarded as joint first authors.

Mol Genet Metab. 2011 Feb; 102(2): 116-21

Contribution: Organization of patient samples, database management, evaluation of the data, proofreading of the manuscript.

II) Genotype-predicted Tetrahydrobiopterin (BH_4)-Responsiveness and Molecular Genetics in Croatian Patients with Phenylalanine Hydroxylase (PAH) Deficiency

Iva Karačić, David Meili, Vladimir Sarnavka, Caroline Heintz, Beat Thöny, Danijela Petković Ramadža, Ksenija Fumić, Duško Mardešić, Ivo Barić, and Nenad Blau

Mol Genet Metab. 2009 Jul; 97(3): 165-71

Contribution: Database management, evaluation of the data, proofreading of the manuscript.

III) Quantification of Phenylalanine Hydroxylase Activity by Isotope-Dilution Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry

Caroline Heintz, Heinz Troxler, Aurora Martinez, Beat Thöny, Nenad Blau

Mol Genet Metab. 2012 Apr; 105(4): 559-65

Contribution: Experimental setup, laboratory experiments, evaluation of the data, manuscript preparation, proofreading of the manuscript.

IV) Splicing of Phenylalanine Hydroxylase (*PAH*) Exon 11 is Vulnerable: Molecular Pathology of Mutations in *PAH* Exon 11

Caroline Heintz, Steven F. Dobrowolski, Henriette Skovgaard Andersen, Mübeccel Demirkol, Nenad Blau and Brage Storstein Andresen

Mol Genet Metab. 2012, in press.

Contribution: Experimental setup, laboratory experiments (except Figure 4.4), evaluation of the data, manuscript preparation, proofreading of the manuscript.

List of Abbreviations

BBB	Blood-Brain Barrier
BH_2	7,8-Dihydrobiopterin
BH_4	(6*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin ((6*R*)-BH_4)
bp	Base pair
CBR	Cofactor Binding Region
cDNA	Complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DBS	Dried Blood Spots
DHFR	Dihydrofolate Reductase
DHPR	Dihydropteridine Reductase
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr Virus
ENU	*N*-ethyl-*N*-nitrosourea
ESE	Exon Splicing Enhancer
ESS	Exon Splicing Silencer
GTPCH	Guanosine Triphosphate Cyclohydrolase
HMG-CoA Reductase	3-hydroxy-3-methyl-glutaryl-CoA Reductase
HPA	Hyperphenylalaninemia
HPLC	High-performance liquid chromatography
ISE	Intronic Splicing Enhancer
ISS	Intronic Splicing Silencer
LC-ESI-MSMS	Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry
LNAA	Large Neutral Amino Acids

MHP	Mild Hyperphenylalaninemia
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NH_2TP	7,8-Dihydroneopterin Triphosphate
NOS	Nitric Oxide Synthase
PAH	Phenylalanine Hydroxylase
PAHdb	Phenylalanine Hydroxylase Locus Knowledgebase
PAL	Phenylalanine Ammonia Lyase
PCD	Pterin-4a-Carbinolamine Dehydratase
PCR	Polymerase Chain Reaction
Phe	L-Phenylalanine
PKU	Phenylketonuria
PTP	6-pyruvoyl-5,6,7,8-tetrahydropterin
PTPS	6-pyruvoyl-tetrahydropterin Synthase
qBH_2	Quinoid-dihydrobiopterin
RNA	Ribonucleic Acid
SR	Sepiapterin Reductase
TH	Tyrosine Hydroxylase
TMS	Tandem Mass Spectrometry
TPH	Tryptophan Hydroxylase
Trp	L-Tryptophan
Tyr	L-Tyrosine

Introduction

1.1 Phenylketonuria and Hyperphenylalaninemia

1.1.1 History of Phenylketonuria (PKU)

In 1930 phenylketonuria (PKU) was acknowledged as an inborn error of metabolism, a concept not widely known at that time. It was the first inherited metabolic disorder found to lead to mental retardation and seriously affecting individuals. PKU is the most common defect in amino acid metabolism and nowadays figures as a model system in genetics, clinical medicine and biology.

The disease was discovered by the Norwegian physician Asbjørn Følling upon analysis of urine from two mentally retarded children presenting with several other clinical symptoms and was later renamed to phenylketonuria (1, 2). The abnormally excreted substance in urine of these children was identified as phenylpyruvic acid, a transamination product from Phenylalanine (Phe). Further investigations lead to the conclusion that the defect is due to elevated concentrations of Phe in the blood. In addition, it was noted that very often siblings were affected, together with consanguinity among parents, suggesting an autosomal recessive inheritance (3, 4).

Phe is an essential amino acid for humans taken up with nutrition. The evidence soon emerged that Phe-low diet could prevent the metabolic phenotype, namely hyperphenylalaninemia (HPA), and thus mental retardation (5, 6, 7, 8). The knowledge that PKU can be treated successfully greatly improved patients' and families' lives. This was also the urge of the bacteriologist Robert Guthrie, successfully trying to develop a more accurate test for PKU and promoting the need to test all newborns, hereby saving many individuals from brain damage. Initially, the detection of alternative metabolites like phenylpyruvic acid was used for diagnosis of PKU using a ferric chloride test (2, 9). Robert Guthrie developed the first practical screening test for PKU in the early 1960s (10). The Guthrie test is a bacterial inhibition assay using a drop of blood from a heel prick spotted on filter paper. The filter paper is applied to an agar gel containing *Bacillus subtilis,* which requires Phe for growth. Bacterial growth only appears in samples with elevated Phe levels and the size of the bacterial colony gives a rough estimate of the Phe concentration in the sample. This test was cheap, reliable and robust. The 'Guthrie cards' are still commonly used today and as Bob Guthrie already suggested, they can be used for testing other metabolic conditions. A newborn screening program for PKU was established in the United States during the late 1960s. Since then, it was further developed and applied in many other countries, improved

with new technologies and expanded to other inherited diseases for the purpose of early diagnosis and treatment.

The enzyme deficient in PKU patients, phenylalanine hydroxylase (PAH, phenylalanine 4-monooxygenase, EC 1.14.16.1) was described in 1953 (11). PAH catalyzes the conversion from Phe to Tyrosine (Tyr) and is mainly active in the liver and kidneys. Since the mapping and cloning of the gene sequence of human *PAH* cDNA in the 1980s (12), much progress in mutation analysis was reported. Finally, a locus-specific database has been set up to maintain and centralize mutation data on *PAH* gene (Phenylalanine Hydroxylase Locus Knowledgebase, PAHdb, www.pahdb.mcgill.ca). Once updated, around 600 mutation records can be found in the continuously expanding PAHdb.

Tetrahydrobiopterin (BH_4) is the essential cofactor in the catalytic conversion of Phe to Tyr (13). In 1974, S. Milstien and S. Kaufman reported on the stimulation of PAH with increase in activity by the addition of exogenous BH_4 added to the medium containing liver slices for the study of PAH (14). H. - Ch. Curtius and A. Niederwieser suggested ten years later that K_m mutants in the *PAH* gene lead to responses to the cofactor and lowering of Phe levels (15) But it was not until 1999 that Kure et al. described patients treated by the oral administration of BH_4. This option significantly changed the field, increased activities in further characterizing BH_4-response and greatly improved treatment for some PKU patients.

PKU emerged as the prototype for treatable human inherited genetic diseases by early diagnosis and showed that a simple Mendelian phenotype can also be a complex disorder (5). The understanding of the links between gene, mutations, enzyme function, metabolism and clinical phenotype provide opportunities to better understand the pathophysiology of disease.

1.1.2 Epidemiology of PKU

The prevalence of PKU varies widely around the world (16). The ethnic background is probably the most predominant factor influencing the incidence of PKU. However, differences in cutoff values and definitions of PKU may lead to inconsistencies in population statistics.

In Europe, depending on the area, the prevalence of PKU is in average 1 in 10’000 live births with highest prevalence in Northern Ireland (1 case in 4’500) and lowest in Finland (1 in 100’000) (17, 18). In the United States, ethnic differences correlate with varying prevalence. The incidence is higher in Caucasians and Native Americans and lower in African Americans, Latinos and Asians (19). This relates to the distribution among those continents of origin (20). Africa has a very low incidence of PKU (21), while in Spain especially mild HPA is predominant (22). In Asian populations, differences in occurrence

can be found within China (between one per 15'000 and one per 100'500) (23, 24), but also Japan and Thailand exhibit lower prevalence than Europe (one per 70'000 and one per 200'000 respectively) (25, 26).

1.1.3 Diagnosis and Classification of Hyperphenylalaninemias

PKU is caused by different disease-causing alleles in the *PAH* gene, and thus deficiency in PAH enzyme function. However, this does not account for all hyperphenylalaninemias and not all degrees of HPA's are identical risk factors for impaired cognitive development (see below, 1.3.2). Treatment of PKU patients by dietary Phe restriction diminishes the toxicity of excess Phe to the brain and enables an almost normal development and cognitive function. Until the mid 1980s, the most specific method in diagnosing and classifying PKU was considered to be the direct measurement of PAH activity in a liver biopsy (27, 28, 29). However, this method is ethically unjustifiably and no longer used.

Today, the diagnosis is based on neonatal screening results of blood Phe levels. Blood Phe concentrations are determined by analysis of dried blood spots (DBS) from a filter paper (Guthrie card) obtained by heel prick, in general around day three after birth. Tandem mass spectrometry (TMS) is nowadays an alternative to the bacterial Guthrie test used for the determination of Phe and Tyr levels from small volumes of blood (30, 31). TMS simultaneously allows the detection of other inborn errors of metabolism, as many amino acids and metabolites can be measured from the same sample. Other fluorimetric and chromatographic screening methods are regularly used as well in PKU newborn screening (32). Blood Phe levels in newborn range between 30 and 120 μM and levels higher than 120 μM are considered as pathological and require further investigation (33). A second test is required for confirmation of HPA and to eliminate the possibility of transient HPA. Delayed maturation of PAH can lead to transiently elevated levels in non-PKU infants (34).

The report by Kure et al. in 1999, which describes several patients to respond to oral administration of the cofactor BH_4 by lowering Phe levels led to a new subgroup in classifying HPA's. Those patients generally present with mild to moderately elevated Phe levels. The diagnosis, properties and mechanisms underlying BH_4 responsiveness will be described in more detail in section 1.7.

After the introduction of newborn screening, PKU became viewed as a complex disorder with variable degrees of severity depending on blood Phe levels and daily dietary Phe tolerance. The recognition that the phenotype varies relative to the degree of HPA led to classification schemes that influence the stringency of treatment (35, 36, 37, 38, 39).

Regulations across countries vary about the start of the treatment, but the following current classification (according to the recent 2012 NIH consensus conference) has been elaborated for the types of PKU (Table 1).

Molecular genetic testing is not primarily performed for diagnosis or treatment, but can be used for genetic counseling, disease classification and genotype-phenotype correlations. The genotype is the main determinant for the metabolic phenotype (40). However, the identification of mutant PAH alleles cannot be used alone for classification of HPA, as many inconsistencies are reported (41, 42). Mutation scanning by denaturing high-performance liquid chromatography and cDNA sequence analysis are methods of choice in molecular genetic testing. New methods like whole genome or exome sequencing are on the way (43, 44, 45).

Table 1: Classification of hyperphenylalaninemias according to blood Phe levels. Blood Phe levels below 120 µmol/L are considered normal (16).

	Blood Phe µmol/L
Mild HPA (no treatment required)	120 - 360
Mild HPA (gray zone for treatment)	360 - 600
Mild PKU	600 - 900
Moderate PKU	900 - 1200
Classic PKU	> 1200
BH_4-responsive HPA/PKU	> 360

PAH deficiency is not always the underlying cause for elevated Phe levels. Upon a positive screening result, further diagnostic tests must be performed in all newborns with even slightly elevated blood Phe levels. In 1 - 2% of HPA cases, mutations in the genes coding for enzymes in BH_4 biosynthesis or regeneration pathways lead to disturbed Phe metabolism. An HPA is confirmed by full amino acid analysis in plasma or blood. The differentiation of a PAH defect and a defect in BH_4 metabolism is done by analysis of urinary or blood pterins and determination of dihydropteridine reductase (DHPR). The differential diagnosis for elucidating BH_4 deficiencies is described in more detail in section 1.3.2.

1.1.4 Clinical Presentation and Pathophysiology

Symptoms of untreated or late-diagnosed PKU patients are generally summarized as mental retardation, but the clinical effects of HPA/PKU are highly variable. Although mental retardation is greatly prevented by an early start of diet, patients treated from birth are often

not completely protected from cognitive impairment. Slowed reaction times, reductions in executive functioning, non-executive functions (speed of information processing, fine motor skills, and perception/visual-spatial abilities), and increased hyperactivity and impulsivity, are among the neuropsychological impairments described in individuals with PKU benefiting from early treatment (46, 47, 48). For example, the IQ in treated children with PKU well reaches average range, but always remains little lower than IQ from siblings and peers. An IQ level reduction was even found to correlate with an increase in Phe levels and leading to a reduced mental processing speed. Children with PKU are often found to perform less well in school (49). Despite a lifelong dietary treatment, some degree of neurocognitive deficit always occurs in most patients with PKU (50).

In untreated or late-treated PKU individuals, clinical symptoms are more severe and range from severe intellectual disability, seizures, ataxia to motor deficits, dermatological and behavioral problems (51). In adults, anxiety disorders and depression have been reported (52, 53, 54). Microcephaly and impaired growth were revealed in adult non-treated PKU patients (55). A characteristic of classic PKU already known from 1930s is a mousy odor that results from the excretion of phenylketone bodies in the urine. Hypopigmentation in PKU patients is a result of deficient melanin synthesis inhibited by the elevated Phe levels (56). Many of these symptoms are only rarely observed nowadays because of the introduction of neonatal screening. Children with a late diagnosis and start of treatment can still markedly benefit from dietary treatment. Cognitive performances may improve in children as well as intellectual functioning, behavior and improved quality of life (57). Nevertheless, intellectual disabilities remain and chronic neurotoxic consequences from high Phe levels are irreversible.

All these symptoms led to the conclusion that a PAH deficiency mainly affects the central nervous system and leads to impaired brain development and function. A broad concordance was found between the metabolic phenotype (determined by the mutant genotype) and the cognitive phenotype (40). *PAH* alleles with a severe effect are likely to cause PKU with low IQ scores in the untreated state, whereas mild *PAH* alleles are likely to cause mild HPA with higher IQ scores (58). Thus, high plasma Phe levels do not always lead to high brain Phe levels and brain abnormalities (59), as shown by noninvasive *in vivo* ^{1}H-magnetic resonance spectroscopy (60, 61). These studies imply that interindividual variation in brain Phe transport is another factor to explain interindividual intellectual abilities in PKU. Some patients with high blood Phe, but normal brain Phe levels just escape brain dysfunction (62, 63).

A general agreement arises that the blood-brain barrier (BBB) plays an important role in neurologic consequences of PKU.

The pathogenesis of brain dysfunction is not completely known. Nevertheless, increased concentrations of Phe in the brain are neurotoxic and cause a disturbance of transport processes of other large neutral amino acids (LNAA), resulting in abnormal homeostasis in the brain. Several mechanisms were analyzed for their involvement in neuropsychological dysfunctioning of the brain (64, 65, 66).

Amino acid transport from blood to brain is a dynamic process, involving a set of amino acid transporters each with specific affinity for a set of amino acids. Phe is entering the brain via binding to the LNAA type 1 transporter (LAT1, SLC7A5). The other LNAA, valine, leucine, isoleucine, methionine, threonine, histidine, tryptophan and tyrosine, are also using this route in a competitive manner (67). In addition, for each LNAA taken into the brain, the LAT1 transporter excretes one LNAA. Moreover, the LAT1 transporter shows highest affinities for Phe among all the LNAA's (68, 69, 70). High concentrations of Phe were found to impair the function of the LAT1 carrier and neuronal uptake of non-Phe LNAA is reduced, leading to low intracellular concentrations of these amino acids. This increases the potential for neurotransmitter dysfunction and their availability for protein synthesis (71, 72). In addition to the high neurotoxic Phe concentrations, deficient LNAA also contribute to pathogenesis in PKU. In general, decreased availability of these LNAA (which are now essential amino acids) results in decreased protein synthesis (73, 74). Regulation of cerebral protein synthesis is essential for brain development and function (75, 76). Myelin is a cerebral protein often found abnormal in PKU and associated with white matter abnormalities detected in PKU patients (77, 78). Reduced myelination was also found in two animal models, in the *Pah*^{enu2} genetic mouse model with PAH deficiency (see also section 1.1.5 below) and in the pharmacologically induced HPA rat model (79, 80). Oligodendrocytes had adopted a non-myelinating phenotype in the animals, which was also confirmed in cell culture (81, 82), possibly appearing secondary to an increase in Phe. Diffuse white matter pathology was found in treated and untreated PKU patients. In untreated patients, this is likely to reflect hypomyelination while in early-treated patients this pathology is likely to reflect intramyelinic edema. Research demonstrated that this pathology is associated with metabolic control and can be reversed with adherence to the strict diet (78). However, impaired myelin function may not completely account for the principal pathophysiological defect in PKU (83).

Oxidative stress is possibly also involved in the pathology of PKU. Causes of oxidative stress are often not well understood. However, it is assumed to result from the accumulation of toxic metabolites leading to the induction of free radical production (84). Oxidative stress is known to be associated with obesity, cardiovascular disease and stress. The low-Phe diet may be a risk factor and lead to deficiencies for example of zinc, selenium, ubiquinone-10 and L-carnitine (85). In addition, the excessive production of reactive species and associated metabolites may increase the risk of oxidative stress of tissue damage in PKU. Several studies in PKU animal models, but also in PKU patients have analyzed oxidative stress parameters (antioxidant enzymes, e.g. Glutathione peroxidase, Superoxide dismutase, catalase, and also non-enzymatic antioxidants, e.g. some vitamins, glutathione, minerals, carotenoids, but also high and low density lipoproteins, summarized in ref. (85)). Especially in late-diagnosed PKU patients, these markers seemed evident, probably associated with the long exposure to high Phe (86). Early diagnosed and diet compliant PKU patients seem more protected from oxidative damage. Further studies in PKU patients are required to confirm results from animal models (87). It is however suggested to maintain patients on follow-up and make them aware of good metabolic control (85, 88).

Figure 1 summarizes different cascades and probable interactions with each other resulting in brain dysfuntion in PKU. Reduced activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) and the function of monoamine oxidase B as a modifying gene were studied in relation with potential pathological mechanisms for HPA-induced damage to the brain (89, 90). HMG-CoA reductase is a rate-limiting enzyme in the metabolic synthesis pathway of cholesterol.

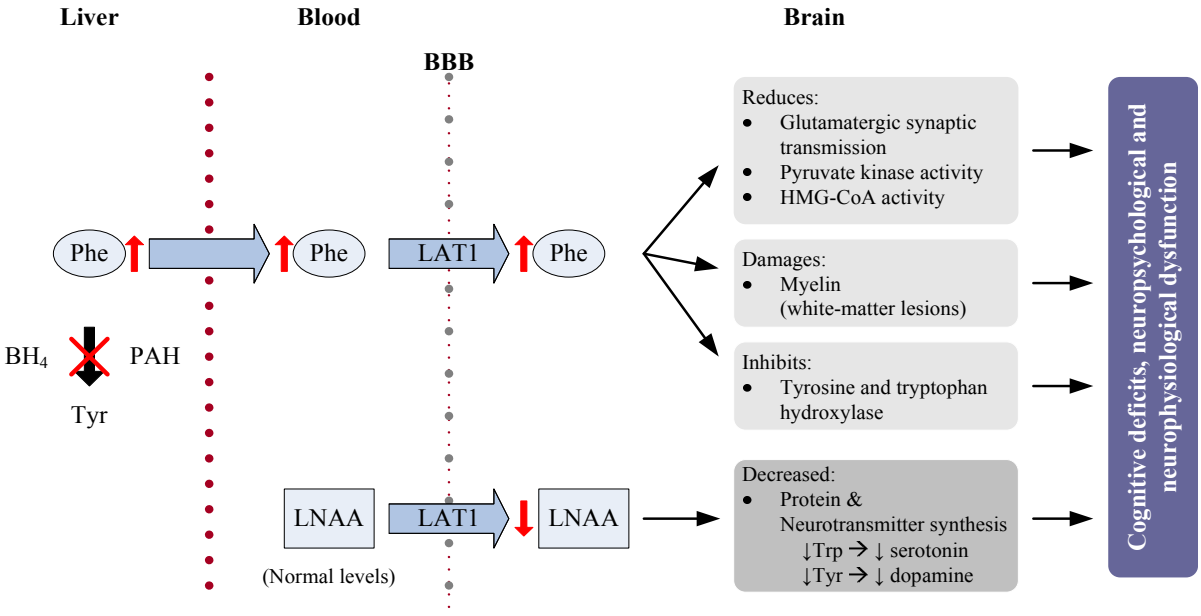


Figure 1: Different cascades and their interaction with each other as a concept of the pathophysiology of brain dysfunction in PKU (Figure modified from ref. (94)).

Myelin, which contains a high content of lipids especially cholesterol, connects these two mechanisms as potential pathogenic factors in brain dysfunction in PKU. It is however not clear, whether impaired cholesterol synthesis leads to reduced myelination in PKU or whether synthesis of HMG-CoA reductase is reduced, suggesting that reduced cerebral protein synthesis may affect enzymes in myelin formation (90). Impairment and reduced activities of several other key enzymes are mentioned, like glutamatergic synaptic transmission, synaptogenesis, and activity of pyruvate kinase, that are still subjects of debate and where the clinical consequences for PKU are poorly understood (91, 92, 93).

Reduced blood Tyr concentrations do not to correlate with cognitive outcome in PKU, and Tyr supplementation alone does not prevent mental retardation (95, 96). However, reduced Tyr and Trp concentrations lead to insufficient synthesis of catecholamines and serotonin in the central nervous system. The metabolic relationship between Phe, Tyr and Trp and their oxidation to neurotransmitter precursors is depicted in Figure 4. Lower levels of dopamine, catecholamine, and serotonin were found in the PKU mouse model P*ah*^{enu2} compared to non-HPA mice. A reduction in the synthesis of these precursor metabolites was also shown in urine and CSF in humans with PKU (97, 98, 99, 100). However, the dietary supplementation of Tyr and Trp restores reduced neurotransmitter synthesis (101). These neurotransmitters are normally concerned with executive functions of the brain and the initiation of movement. In addition, cerebral serotonin deficiency may explain the increased occurrence of anxiety and depression disorders in PKU patients. High Phe concentrations were even shown to inhibit the activity of the other two monooxygenases TH and TPH (102, 103, 104). In contrary, Fernstrom and Fernstrom (105) negate high Phe levels as inhibitors for TH and these findings alone are still matter of debate. Burlina et al. showed, that in untreated PKU patients, i.e. at high blood Phe levels, both dopamine and serotonin metabolites are extremely low in CSF (106). In summary, although dopamine and serotonin are involved in postnatal brain development and maturation, these findings do not explain the severe mental retardation of PKU patients, but they are likely to explain certain cognitive deficiencies.

1.1.5 Treatment Options for PKU

Since the discovery of Phe-low diet, resulting in near-normal cognitive development in PKU, dietary management has emerged as effective treatment for 60 years. It is an easy and straightforward treatment. Nevertheless, alternative strategies are required and advancing, as adherence to natural protein restriction is often compromised at several stages of life. In addition to dietary food, other nutritional deficits may occur through PKU resulting in

variable neurological outcomes (94, 107). Diet imposes a burden on patients and families, economical and social, which is a downside of the therapy (108) and compliance is often a problem (109). Reasons reported for low compliance with diet include lack of time, impact on social life and stress associated with food preparation (110, 111).

It is still debated whether plasma Phe concentrations lower than 600 µmol/L require dietary treatment (63, 112). These individuals were not found to be at higher risk for developing neurological impairment than non PAH-deficient individuals. In addition, Phe levels often change between newborn and later age, so that the diet should also be adjusted according to stage of life. Phe can only reach its full potential biological value in non-restricted dietary status, which has to be considered when analyzing test results. The Phe level during dietary treatment is often considered as a tolerance level and an estimate for residual PAH.

The main focus of new treatment approaches is the decrease of the blood Phe levels. The increasing knowledge on the genetic basis of PAH deficiency has allowed for the investigation of novel pharmacologic therapies to directly ameliorate the effects of the mutant enzyme (113). PKU is targeted at the different levels, from nutritional intake and intestinal uptake to the liver and the BBB, where toxic effects of high Phe levels have been observed. The dietary management, as well as promising alternatives in development will be discussed in the following paragraphs.

Relevance of PKU animal models

PKU animal models provide a great basis for characterizing different aspects of Phe metabolism and kinetic properties of PAH protein. Many early studies were performed in a pharmacologically induced rat model (79), but a better opportunity to obtain a mammalian counterpart of human PKU now exists in mice. The access to a model of the human disease in an orthologous animal has greatly advanced work of therapies for PKU (114). Two homoallelic and one heteroallelic orthologous mouse models are frequently used in the pre-clinical investigations: $Pah^{enu1/1}$ (ENU1) and $Pah^{enu2/2}$ (ENU2), and the hybrid strain $Pah^{enu1/2}$ (ENU1/2), respectively. The models have been created by chemical mutagenesis with the ENU mutagen (*N*-ethyl-*N*-nitrosourea) (115), which induces point mutations. Mice with various Phe levels were found, corresponding to mild and classic PKU phenotypes. The homozygous ENU1 mouse is classified as non-PKU HPA type (116), whereas the ENU2 mouse exhibits a PKU phenotype with no PAH activity (117), very similar to human classical PKU. The mutations were characterized later by DNA sequence analysis (118). The $Pah^{enu1/1}$ mutation was identified as c.364T>C leading to p.V106A missense change on protein level.

The *Pah*$^{enu2/2}$ model harbors the c.835T>C (p.F263S) mutation. In the human *PAH* sequence, this mutation corresponds to p.F263L (c.789C>G) amino acid substitution as was once reported in a PKU patient (119). The ENU1/2 mouse orthologue is hyperphenylalaninemic with moderate, intermediate phenotype of the other two strains (p.V106A/p.F263S) (120). With these three models, all phenotypic levels of human PKU can be represented. The heteroallelic ENU1/2 model was reported particularly useful for the assessment of alternative therapies for PKU (120) and pharmacological chaperone mechanism, as it is also a model for BH_4-responsive PKU (121, 122).

Dietary therapy

The substantial effect of dietary restriction is due to the fact that Phe is not synthesized in the body and that the concentration of Phe in the blood can be controlled by dietary intake. Although there are no common dietary guidelines around the world, consensus is found that dietary therapy is started early after the positive newborn screening results and that treatment in infancy is highly important (123, 124).

Phe is present in all naturally existing proteins. This means that meat, fish, cheese, eggs, seeds with flour, milk, tofu or soya for example cannot be readily eaten or even need to be completely avoided, depending on the type of severity. The protein content of vegetables and potatoes needs to be analyzed before consumption. Foods are classified according to their Phe content and the amount of each to be eaten can be calculated. But this also means that protein substitutes need to be consumed with the food for avoiding other nutritional deficits. Much progress has been made in those chemically manufactured food mixtures to provide good taste and smell and all nutritional components required, but they might never reach the status of normal food. This is another drawback on treatment of PKU making it less palatable and reducing good compliance over a long period of time.

The stringency of the diet can vary widely, according to the severity of the PAH deficiency and the stage of life. In general, cutoff values rise from the age of 4 or 8 and later in adolescence with an upper limit of 600 μmol/L accepted in some countries. But individual goals are often set (see Table in (16)). Constant blood Phe monitoring and intensive guidance by a healthcare team, as well as training for self-management, follow-up and good communication with the patient belong to successful approaches for treatment of PKU.

There is a rationale from several studies for lifelong treatment to minimize neurological damage that can still occur at later stages of age. Also upon discontinuation of treatment, severe symptoms can return like seizures and anxiety disorders. Restoration of metabolic

control ameliorates these symptoms and improved emotional functioning and quality of life were noticed (125).

Treatment with large neutral amino acids (LNAAs)

All non-Phe LNAAs become essential amino acids for the brain in a PAH-deficient individual when Phe blocks transport across BBB and need to be provided through other pathways. As these amino acids share common transport mechanisms with Phe, several hypotheses emerged for beneficial LNAA supplementation. These ideas have been investigated already early on (126). LNAA administration was shown to reduce toxic Phe levels in the brain of patients despite constantly elevated serum concentrations (71, 127, 128). In this way, other amino acids with high affinity for the BBB transport system keep high plasma Phe concentrations from entering the brain. This strategy could help to protect the brain in non-compliant patients. In addition, LNAA supplementation was shown to result in improvement of neuropsychological functions without decrease of cerebral Phe concentrations (129, 130). Higher doses of Tyr and Trp also lead to increased synthesis of dopamine and serotonin in HPA patients (101). The effect of LNAA is also influencing intestinal tract transport, as decreased blood Phe concentrations were determined upon supplement administration (129).

These supplements may not replace Phe-low diet, but may help to relax dietary restriction of treated individuals or help in management of untreated adults with classic PKU. However, further clinical trials and data are required to examine safety and efficacy of LNAA therapy still in development.

Glycomacropeptide

Another strategy for targeting the intestinal tract by development of medical food is supplementation of glycomacropeptide. Glycomacropeptide is a natural protein from cheese whey that is rich in specific essential amino acids but contains no Phe, Tyr, or tryptophan (Trp) (131). Food enriched with this protein and supplemented with Tyr and Trp can be given as an additional measure and patients may need less amino acid mixture. However it needs to be assured that purity is sufficient and that it is absolutely free of Phe. Studies have given promising results in terms of palatability, safety and improved compliance, but further evidence is still required (132, 133, 134).

Enzyme substitution therapy

Enzyme therapy can be addressed in two different ways, either by replacing PAH or by substitution with a foreign protein capable of metabolizing Phe. Replacement of PAH is highly challenging, as the whole, intact multi-enzyme complex for PAH catalysis including

BH_4-cofactor is required. In addition, PAH regulation is complex and high amounts of stable PAH are tedious to isolate and purify.

The enzyme of choice for substituting PAH is phenylalanine ammonia lyase (PAL, EC 4.3.1.5) (135, 136), derived from plants and compared to PAH, sufficiently more stable as oral formulation. PAL targets the intestinal system and is also involved in Phe metabolism, capable of lowering blood Phe in humans. This exogenous enzyme converts Phe to *trans*-cinnaminic acid and negligible amounts of ammonia without the need of a cofactor, making it catalytically less complex than PAH. In humans, *trans*-cinnaminic acid is safely and rapidly converted to hippuric acid, which is then excreted in the urine (137, 138).

The efficacy of PAL was tested in the PKU mouse model ENU2 (see above) and yielded complete correction of blood Phe concentrations in blood and brain with a sustained effect for up to one year with weekly injections (139). The optimal source of PAL was later found in the algae *Anabaena variabilis* (140). The PEGylation (attachment of polyethylene glycol polymers to lysine side chains) diminished immune-mediated detection and elimination of the injected enzyme (141). Clinical trials have been initiated in PKU patients and no severe adverse events were recorded, but a significant decrease in Phe blood levels after a single injection. A phase II clinical trial is in progress in addition to development of an orally administrable PAL form (142). PAL would probably be used in addition to a less stringent Phe diet.

Gene therapy

Gene therapy approaches for treating PKU could be a permanent therapy, which is highly desired for alleviating the need of complicated diet. Unfortunately, PKU can only be a candidate when gene therapy is safe and an efficient delivery system for lasting correction of PKU phenotype is developed. Promising investigations in PKU murine models are on their way, but still several years away from clinical trials in humans. Recently, much success has been achieved with use of recombinant adeno-associated viral (rAAV) vectors for gene transfer targeting the liver in correcting HPA for up to one year in mice (143, 144). However, the vector's genome is gradually eliminated as it is not integrated into the hepatocyte's DNA and re-injection was not effective due to immunological responses.

Another attractive target organ for gene therapy is skeletal muscle. It is easily accessible and does not undergo cell division. For efficient PAH metabolism, local production of BH_4 is required and so enzymes involved in BH_4 metabolism need to be expressed likewise. A promising outcome of this strategy of mimicking the role of a second phenylalanine metabolism has also been reported (145).

Liver- and muscle-directed gene therapy need to be further optimized with improved sustainability (146). Promising results were found upon application of non-viral gene therapy with increased efficacy and less adverse effects (147). Successful gene therapy might then lead to human trials in PKU in the next few years. Not only *PAH* gene transfer, but also therapeutic liver repopulation with wild type hepatocytes and even liver transplantation have been investigated as potential approaches for treating PKU (148). Clinical evaluations for these treatment alternatives are still required.

Tetrahydrobiopterin (BH_4)

An oral BH_4 loading test was introduced in the 1980s to discriminate between patients with PAH deficiency or with a BH_4 deficiency (149). With BH_4 formulations of higher purity (only 6*R*-BH_4), doses in the loading test were increased from 7.5 to 20.0 mg/kg body weight. Kure et al. (150) reported in 1999 a group of patients carrying mutations in the *PAH* gene with decreasing blood Phe values several hours after oral administration of the PAH cofactor BH_4. Around 30% of patients with PKU are now thought to respond to BH_4 treatment (151), and there is wide range in the extent of response. While the most rapid Phe reduction of up to 90% after 4 - 8 hours is highly indicative for a BH_4-deficiency, in PKU patients responding to BH_4 administration, blood Phe reduction of 30 - 80% is evident after 24 - 48 hours. Therapy with BH_4 as an alternative approach to the Phe-low diet was introduced in 2001 (152).

Different test protocols have been developed for the oral loading test as well as different guidelines among countries for time of testing and cutoff levels. This again leads to heterogeneity among loading test results and inconsistencies in predicting response, justifying requirement of further testing. BH_4-responsive patients generally present with milder forms of PKU, which is also illustrated from several studies, displayed in of Figure 2. Panel A shows the outcome of the 24-hour loading test in European PKU population with 20 mg/kg BH_4 and a 30% responsiveness cutoff (A). In B, a number of patients in the USA who responded to commercial BH_4 formulation sapropterin (10 mg/kg per day) on day 8 with at least a 30% reduction of blood Phe concentrations are displayed. The proportion of BH_4-sensitive patients increases as the severity of the phenotype decreases (153), which is due to the mechanistic action of administered BH_4, requiring sufficient active hepatic PAH protein (residual enzyme activity). However, this eliminates the need for challenging every patient. The mechanistic details and explanations for BH_4 responsiveness are described in section 1.7. Two different forms of BH_4 have been used in clinical investigations. Since 2008, sapropterin dihydrochloride (Kuvan® or Biopten®, BioMarin Pharmaceutical Inc), the synthetic analogue

of BH_4, has been market approved for treatment and is commercially available in the US, Japan and Europe, whereas the older form, 6*R*-BH_4 dihydrochloride is unregistered (Schircks Laboratories, Jona, Switzerland). However, both forms are chemically identical.

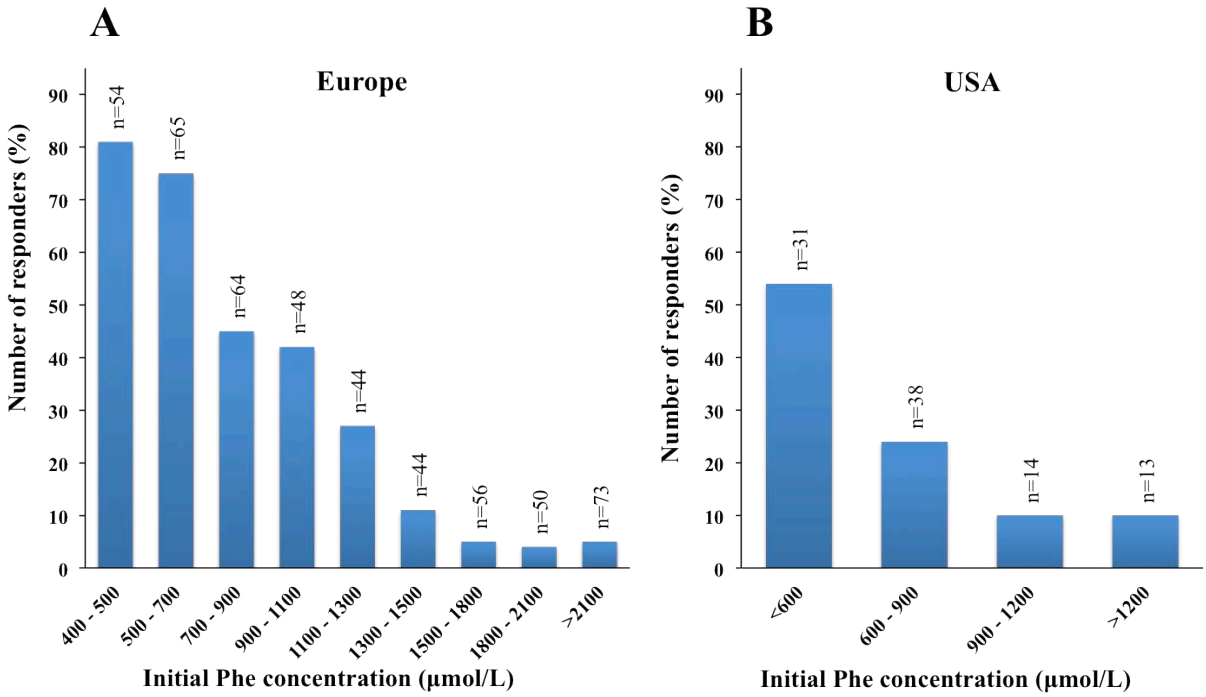


Figure 2: Responsiveness to tetrahydrobiopterin in patients with phenylketonuria according to initial blood phenylalanine concentrations (Figure adapted from ref. (16))

Generally accepted as the threshold of BH_4 responsiveness is a reduction of blood Phe levels of at least 30% during the first 8 - 24 hours after oral administration of BH_4. Test durations may be even up to 4 weeks. Several late responders can be detected in longer test periods. The test dose is typically 20 mg/kg bodyweight and is administered once a day with regular blood sampling. The detailed comparison of test protocols is described elsewhere (154).

In clinical trials, BH_4-treatment was mostly well tolerated with no severe side effects and long-term efficacy was found. A successful treatment leads to a higher Phe tolerance and allows these patients benefit from a relaxation of the strict diet. A modification of the diet and constant monitoring of Phe values is, however, required. Eventually, patients with mild phenotypes and good responsiveness can attain a normal unrestricted diet (155, 156, 157, 158). Responsiveness testing and treatment with BH_4 is rather expensive and not available for all patients. This might change in the future when the prediction of response to BH_4 treatment is ameliorated. Some *PAH* mutations could be associated with a BH_4-responsive phenotype (159, 160) and a combination of genotyping with the BH_4 challenge may be the method of choice for prediction of BH_4 responsiveness. The online BIOPKU database

(www.biopku.org) tabulates available data on almost 800 genotypes, phenotypes and BH_4-response in patients previously tested and is used as a reference tool in consulting whether to challenge a patient based on previous similar genotype results.

Pharmacological Chaperones

BH_4 induces a gain-of-function in PAH in a multifactorial response mechanism with a notable stabilization of PAH structure (subchapter 1.7). However, efficacy studies of treatment with BH_4 revealed that it is not able to significantly lower blood Phe levels in all milder PKU genotypes and other potential ligands as therapeutic agents are investigated. Pharmacological chaperones are compounds of low molecular weight that promote folding by stabilizing the native state of a target protein without actually binding to them, or by facilitating the folding of nonnative intermediates (161, 162). The pharmacological chaperone-induced stabilization of protein conformations corrects protein misfolding otherwise leading to loss of function. They showed their potential in the treatment of several genetic disorders in successfully reducing clinical symptoms, for example in lysosomal storage diseases and cystic fibrosis. Pharmacological chaperones are either developed by optimization of natural or synthetic substrates with increased affinity or by screening procedures. Two screening studies revealed several promising candidates for further drug development. Pey et al. employed a high-throughput screening of a thousand compounds by fluorescence monitoring for PAH stabilizing ligands (163). The efficiency was demonstrated in cell and animal models and the molecules present a basis for drug optimization. Santos-Sierra et al. performed a virtual screening in a chemical library to identify potential candidates for further testing *in vitro* and *in vivo* in a PKU mouse model (164). Their results present another class of molecules for further development into a more effective drug treatment for PKU.

1.2 The Phenylalanine Hydroxylase System

1.2.1 Aromatic Amino Acid Monooxygenases in the Mammalian Phenylalanine Metabolism

The concentration of Phe in the blood is determined by dietary intake and the balance between protein synthesis and catabolism (including free amino acid pool) (165). The hydroxylation to Tyr through PAH is the limiting step and major pathway for catabolizing dietary Phe. In addition, it is the only metabolic pathway through which Phe can be completely catabolized to carbon dioxide and water (166).

PAH gene expression is observed at the transcriptional and translation levels not only in liver, but also in the kidney. Up to 45% of enzyme activity, compared to the liver, were reported in the kidney and therefore, PAH function also plays a physiological role in this organ and adds to the phenotypic heterogeneity in PKU (167). It has been shown that kidney can influence Phe and Tyr homeostasis as well (168, 169), although slight differences in activation mechanisms and enzyme conformation were found (166).

PAH is an iron-containing enzyme depending on 6(*R*)-L-*erythro*-tetrahydrobiopterin (BH_4) as cofactor and molecular oxygen for efficient catalysis. During the reaction, BH_4 is oxidized to 4a-hydroxypterin and needs to be regenerated for continuous catalysis (Figure 3). The regenerating enzymes pterin-4a-carbinolamine (PCD) and dihydropteridine reductase (DHPR) complete the phenylalanine hydroxylating system (170).

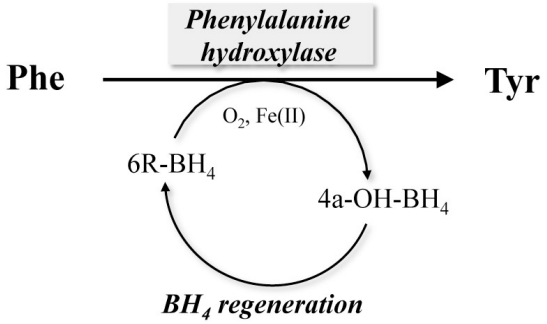


Figure 3: Overview of the reaction catalyzed by phenylalanine hydroxylase (Figure adapted from ref. (154))

The phenylalanine hydroxylating system provides an endogenous source of Tyr, making Tyr a non-essential dietary component (171). PAH together with tyrosine hydroxylase (TH, EC 1.14.16.3) as well as tryptophan hydroxylase (TPH, EC 1.14.16.4) constitute a family of aromatic non-heme iron monooxygenases that use BH_4 as cofactor (172, 173). All three of them are involved in metabolic pathways critical for normal functioning of the nervous

system (170) with similar catalytic and structural properties as well as high sequence similarity. The C-terminal region of the three hydroxylases (monooxygenases) is more conserved, containing the determinants for hydroxylating activity and BH_4 binding, whereas the N-terminal domains are more divergent leading to the different substrate specificities (174). TH catalyzes the hydroxylation of Tyr to L-dihydroxyphenylalanine (L-DOPA), whereas Trp is oxidized to 5-hydroxy-Trp by TPH (Figure 4). The subsequent decarboxylations of L-DOPA and 5-OH-Trp are catalyzed by the aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28).

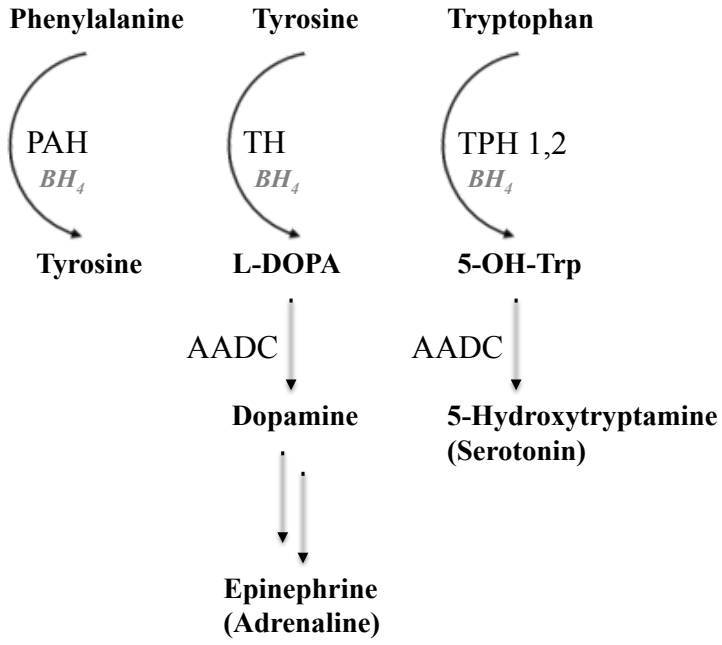


Figure 4: BH_4-dependent hydroxylation of aromatic amino acids by the aromatic amino acid monoxygenases. The next step reaction is catalyzed by aromatic amino acid decarboxylases (AADC).

The biosynthesis of the neurotransmitter monoamines dopamine, norepinephrine and serotonin is dependent upon the availability of the precursor amino acids Tyr and Trp within the brain and the presence of a normal Phe concentration (64). A defect in PAH system leading to excess Phe and a relative lack of Tyr and Trp in the brain has consequences for the subsequent enzymatic reactions. High Phe concentrations compete with the transport of other LNAA through the BBB and lead to a lack of reaction products of Phe, Tyr and Trp. Tyr and Trp now become essential dietary components required for the synthesis of dopamine and serotonin.

In PAH deficiency, accumulating Phe is partially degraded through alternative pathways. Derivatives formed by transamination and decarboxylation reactions are excreted through urine and can be detected in non-treated PKU patients. Phenylpyruvic acid, obtained through

transamination of Phe is a phenylketone from which the name of the disease was derived. Initially, the detection of these alternative metabolites was used for diagnosis of PKU using a ferric chloride test (2, 9). However, phenylketone accumulation can be delayed and is even not observed in partial deficiencies of PAH with low degrees of blood Phe elevation resulting in non-PKU HPA. Figure 5 describes possible breakdown routes of Phe with major inputs and runouts. Phe decay can take place via three major routes: (1) by hydroxylation to Tyr (catalyzed by PAH, and followed by oxidation); (2) by incorporation into bound (polypeptide) pools; and (3) by the chemical reaction pathways of transamination (A) and decarboxylation (B). The transamination pathway becomes only functionally significant when the Phe concentration is much increased.

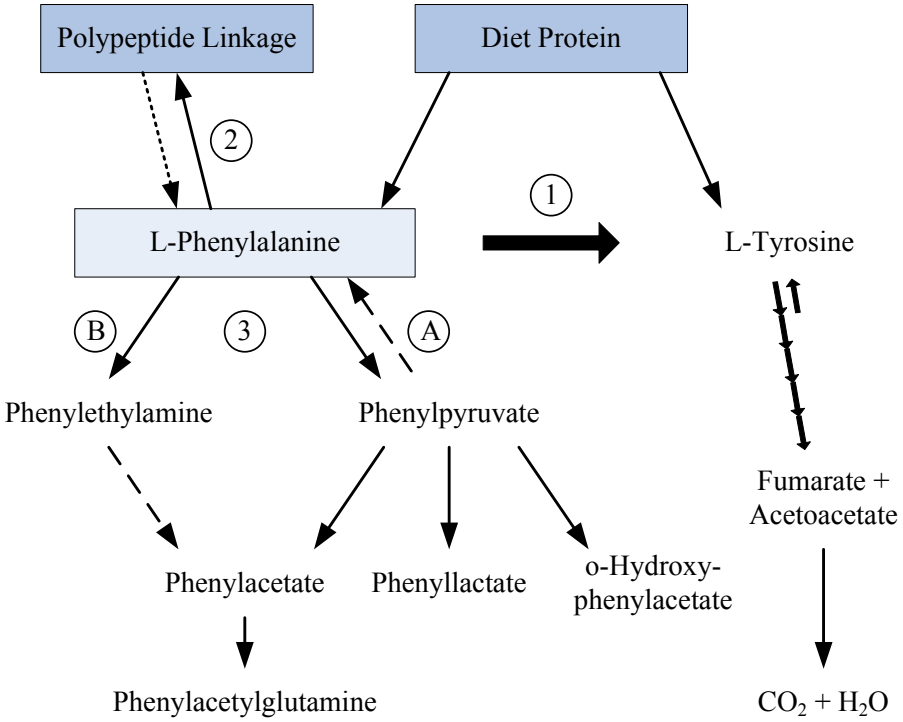


Figure 5: Major inputs and runout of free Phe in human metabolism (Figured based on ref. (175)) ⟶ : major reaction.

The oxidation of Phe to Tyr and further combustion to CO_2 and H_2O are the only possible reactions by which the phenyl ring can be ruptured (166). Nevertheless, under normal conditions, the conversion of phenylalanine to non-tyrosine derivatives constitutes only a minor alternative. In case of inadequate nutrition for example, protein catabolism occurs and free Phe is released. But these endogenous peptide-bound pools contribute only little to the input of plasma Phe.

Another consequence of PAH deficiency is a decrease in melanin synthesis by the enzyme tyrosinase (EC 1.14.18.1), which uses Tyr as substrate (56). In addition, tyrosinase is competitively inhibited by high Phe concentrations.

1.2.2 The *PAH* Gene and the Diversity of Mutations

The human phenylalanine hydroxylase gene (*PAH*) is located on chromosome region 12q22 - 12q24.2 containing the nucleotide sequence coding for the hepatic enzyme phenylalanine hydroxylase (PAH, phenylalanine 4-monooxygenase, EC 1.14.16.1). The *PAH* gene sequence consists of 13 exons and their requisite introns (12), adding up to 171 kb including the flanking regions, encoding a 51.9 kDa polypeptide sequence with 452 amino acids. The exonic sequences in *PAH* take up less than 1% of the genomic sequence. The complete sequences have been catalogued in GenBank under NM_000277 (mRNA, 2680 bp), U49897.1 (cDNA, 1359 bp), AF404777 (gDNA, 171 kb) and NP_000268.1 (protein, 51.9 kDa) or in Ensembl under the reference number ENSG00000171759.

The *PAH* gene is the only gene associated with PAH deficiency, which results from mutations in both alleles disturbing enzyme function. Isolation and sequencing of PAH cDNA (176, 177) opened new fields for intense exploration of the molecular causes of PKU. Over 550 pathogenic mutations have been detected distributed over all 13 exons as well as in introns and flanking regions. The phenotypic severity of PKU is characterized by the type of mutation, and thus by residual enzyme activity. An important resource for information on allele variants is the online relational locus-specific knowledgebase *PAH*db, originated in the *PAH* Mutation Analysis Consortium (178, 179). The database provides information on mutations and associated phenotype, gene and enzyme structure, relation to other species, clinical guidance and much else, reported by clinicians and laboratories from around the world (180). Since the latest updates of this database roughly 61% of all mutations listed are missense mutations, followed by small deletions (13%), splice mutations (11%), silent and nonsense mutations (5 - 6%), and small insertions (2%).

Next to the disease-causing alleles, the *PAH* gene contains at least 25 recognized non-pathogenic single nucleotide polymorphisms (SNPs) with many probably still unrecognized. Multiallelic tandem repeat sequences and biallelic restriction fragment length polymorphisms (RFLPs), embedded in the genomic sequence, provide signatures of multiple haplotypes with particular associations for a wide range of disease-causing mutations. Repeat sequences like tetranucleotide short tandem repeats (STR), variable number of tandem repeats and *Alu* repeat elements are also annotated on the gDNA sequence, that all served to describe haplotypes, facilitate population genetics and led to discovery of large deletions (180).

The DNA and amino acid sequences of mouse, human and rat are highly similar with over 90% identity on protein level (181). Mouse and rat PAH proteins consist of 453 amino acids, which is one amino acid longer than human PAH and differ in ten amino acids,

compared to 36 amino acid variations between the mouse and the human PAH. Even though physiological and regulatory differences were reported, PAH from rodents served as an efficient basis for animal studies (166, 182).

1.2.3 Structural Basis and Regulation of Phenylalanine Hydroxylase

The success of a large-scale production of recombinant human PAH in 1995 (183) finally led to the crystallization of a truncated form of the enzyme. The rat liver PAH structure had already been reported (184), but could not be used for correlating structure and function due to molecular heterogeneity compared to the human version. Until now, the best model available is a composite model, as the crystal structure of the fully active form of PAH is still not yet available.

Human PAH is a cytosolic protein and exists in solution as a pH-dependent equilibrium between functional tetramers and dimers (185, 186) (which also accounts for recombinantly produced human PAH (183)). The composite model of the tetramer is depicted in Figure 6 panel A, assembled from several truncated forms that were structurally characterized. A shift from predominantly dimer to predominantly tetramer was found in the presence of Phe (183, 187). Like the other two aromatic amino acid hydroxylases TH and TPH, mammalian PAH consists of three domains: the regulatory domain (residues 1-142) at the N-terminal part of the protein, the catalytic domain (residues 143-410) and the tetramerization domain (residues 411-452) (Figure 6 panel B). The model was generated using the structures of catalytic/tetramerization domains (188, 189) and regulatory/catalytic domains (190) and superimposing the catalytic domains of the two models.

The catalytic and C-terminal domains of the three hydroxylases are highly homologous (191). The catalytic domain of PAH contains the active site with the iron center and the binding sites for Phe and BH_4. The secondary structure of the tetramerization domain is essential for dimer and tetramer formation. The C-terminal domain of PAH is involved in subunit-subunit interactions leading to a dimer of dimers associated asymmetrically (189). In the dimer, the two catalytic domains closely interact with each other and with one of the regulatory domains. In order to maintain Phe homeostasis *in vivo*, PAH is highly sensitive to changes in substrate concentrations and its activity is tightly regulated. In normal cases, the exposure of tissues to high levels of Phe should be limited, but in parallel, Phe stores should not be depleted in order not to compromise protein synthesis.

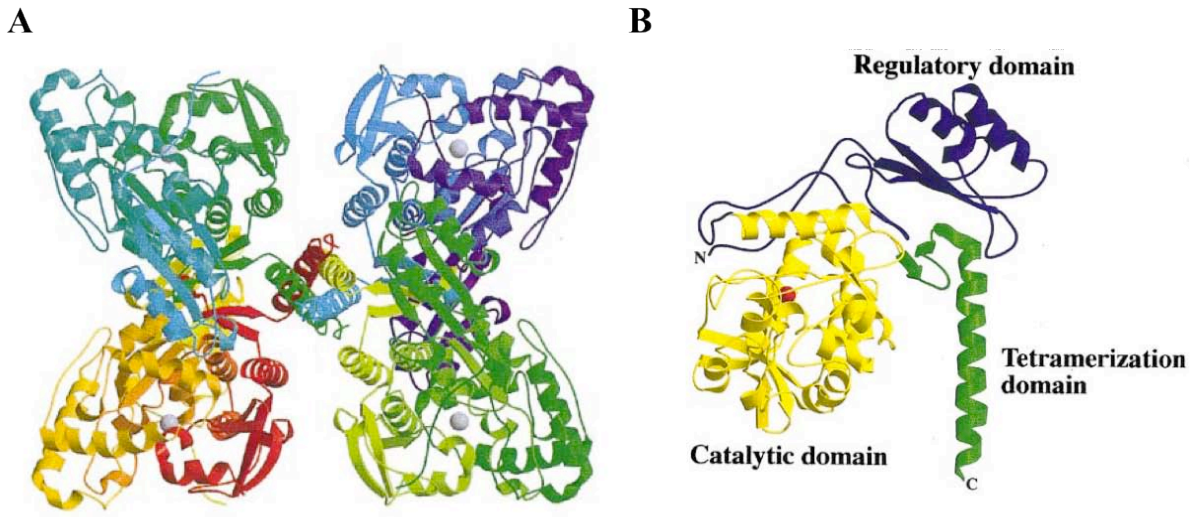


Figure 6: (A) Perpendicular view of the full-length human PAH model structure, colored from red (N-terminus in monomer A) to blue (C-terminus in monomer D). The iron is shown as a gray sphere in all four monomers. (B) Structure of human phenylalanine hydroxylase full-length composite model. The regulatory domain is shown in purple, the catalytic domain in yellow, and the tetramerization domain in green. The iron is shown as a red sphere. (Structures from (192))

The molecular mechanisms described for this regulated enzyme are reversible phosphorylation, substrate activation and relevance of the cofactor (175). The first 30 residues of PAH act as an autoregulatory sequence that includes the Ser16, a substrate for cAMP-dependent protein kinase (193, 194), as well as is essential for the expression of positive cooperativity induced by the substrate and the inhibitory effect caused by the natural cofactor BH_4 (190, 195, 196). This sequence region is termed autoregulatory, as it sterically limits substrate access unless the enzyme is activated by Phe binding to the regulatory domain. The properties of the complex PAH regulation were intensively studied from the 1970s till the 1990s by Seymour Kaufman and Ross Shiman (166, 197, 198). Purified rat liver enzymes were initially mainly used, for availability reasons (185, 199).

The activation by the Phe substrate results in a significant increase in the initial rate of Tyr formation and positive cooperativity in response to the Phe concentration was found (200). Activation and cooperativity are probably related to a conformational change induced in the PAH protein upon substrate binding that alters the spectroscopic properties (187). One monomeric PAH unit contains only one binding site for Phe, located in the catalytic domain (201). These reversible conformational changes upon binding are transmitted throughout the enzyme, displacing the autoregulatory sequence and leading to the propagation of the activating process to the adjacent subunit in the dimer and finally to the other dimer through the oligomerization domain (202). In contrast to Phe, the cofactor BH_4 acts as an allosteric inhibitor, keeping the enzyme in a low activity state and blocking the substrate-activating conformational change (166). BH_4 interacts with the N-terminal autoregulatory sequence and

leads to a dead-end PAH-BH_4 complex, closing the entrance to the active site and leaving the enzyme in a latent, low-activity state (198, 203, 204). Phe binds with lower affinity to PAH than the cofactor BH_4 (205). The high affinity binding of BH_4 and the inhibitory regulatory effect in non-Phe activated PAH are associated with specific interactions of the BH_4 side chain with residues from the catalytic and regulatory domain (203). Several analogs of BH_4 have been shown to effectively catalyze the PAH reaction, but with much lower affinity and only BH_4 is a negative regulator of the PAH system (183). Novel studies with a continuous PAH assay (206), as well as with a high-throughput isothermal titration calorimetry-based activity assay (207), have further characterized BH_4 kinetics and showed that PAH activation by Phe leads to positive cooperativity in BH_4 binding. A Hill kinetic model displays a better fit than previously reported Michealis-Menten kinetics. As a result of cooperative BH_4 binding, different conformational alterations propagating cooperativity than found for Phe binding were reported. An influence of the patient's metabolic phenotype may therefore have a significant effect in drug response. These results are important in developing the oral therapy with BH_4. Several mutations in these regions involving sensitive PAH regulation can lead to different interactions and activation patterns and result in varying hydroxylation mechanisms.

The iron atom is also essential for catalytic activity (208). It is coordinated to two histidine residues (H285 and H290), as well as to one oxygen from E330. The rest of the coordination sites of iron are occupied by water molecules, which are all displaced upon substrate and cofactor binding. This leaves an open coordination site for the reaction with O_2 and generates an activated intermediate in the hydroxylation of the Phe and BH_4 (209).

The composite model of full-length PAH provided an important basis for analysis of the numerous mutations resulting in deficient PAH activity. These structures lead to the elucidation of the catalytic mechanism (210, 211, 212), and the explanation of the regulatory behavior towards the substrate, the cofactor and phosphorylation (201, 203, 204, 213, 214). In addition, 3D structural studies and mapping of mutations can help predict the likely effects of a particular mutation upon protein structure or function in some cases, or generate hypotheses which can then be further tested by functional assays. Many mutations detected in PKU patients were tabulated according to their location and predicted effects on protein structure. If available, the structural changes are compared to *in vitro* expression data and phenotype information. *PAH* mutations were classified in five categories according to their location in the monomer: active site mutations, residues at the dimer or tetramer interfaces, deletion and insertion mutations, interdomain structure mutations and residues interacting

with the N-terminal autoregulatory sequence (192, 215). Most recently, the evaluation of BH_4-responsive PKU/HPA was greatly aided by the analysis of relevant mutations in a structural context (216, 217). The relevant structural details for mutations associated with BH_4 responsiveness will be further discussed in section 1.7.

1.3 The Cofactor Tetrahydrobiopterin (BH_4)

1.3.1 Cofactor Biosynthesis and Functions

BH_4 belongs to a group of chemicals known as the pteridines, natural heterocyclic compounds widely distributed. Gowland Hopkins first reported them at the end of the 19th century in relation to the wing pigment in certain butterflies (218). The pteridines are the molecular basic moieties of the pterins, flavins and folates, which are substituted derivatives commonly found in biomolecules in different forms depending on the function (Figure 7 panel A). Substitution with a dihydroxy propyl chain gives the biopterin molecular skeleton (Figure 7 panel B). The pteridine ring systems can occur in several different oxidation states, i.e. in the fully oxidized, dihydro, and tetrahydro forms. But only the fully reduced biopterin form i.e. BH_4 exhibits biological activity.

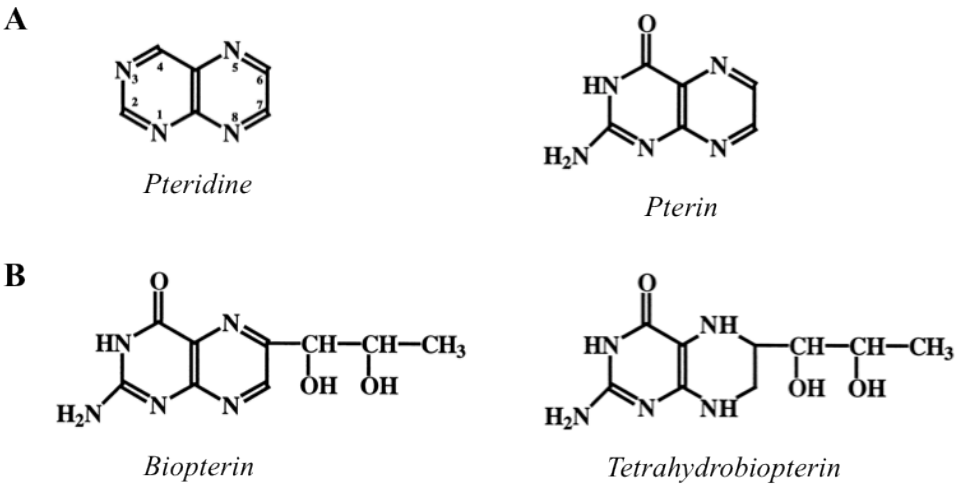


Figure 7: A: Basic structures of pteridine and pterin; B: Biopterin and Tetrahydrobiopterin (BH_4) (Molecular structures adapted from ref. (219))

BH_4 is the short name of its correct chemical name 2-amino-4-hydroxyl-6-[L-erythro-1', 2'-dihydroxypropyl]-tetrahydrobiopterin (13). BH_4 is essential for many diverse processes and ubiquitously present in all tissues of higher organisms. The *de novo* synthesis of BH_4 is a highly regulated process that restores the oxidized forms. It is synthesized endogenously in several enzymatically-controlled steps from guanosine triphosphate (GTP) via two pterin intermediates (Figure 8). The enzyme that catalyzes the conversion of GTP to 7,8-dihydroneopterin triphosphate (NH_2TP) is GTP cyclohydrolase I (GTPCH I, EC 3.5.4.16) in

a Mg^{2+}, Zn^{2+}, and NADPH-dependent reaction (220). This primary step is rate limiting and GTPCH-activity is regulated through several factors in both directions on transcriptional and post-translational levels. These include proinflammatory cytokines and hormones. In Phe metabolism for example, the GTP cyclohydrolase feedback protein (GFRP) exerts feedback inhibition by BH_4 (negative) and feedforward stimulation by Phe (positive) through complex formation (221, 222). This leads to the physiological consequence of high BH_4 plasma concentrations in HPA patients (223).

The subsequent conversion of NH_2TP to 6-pyruvoyl-5,6,7,8-tetrahydropterin (PTP) is catalyzed by 6-pyruvoyl-tetrahydropterin synthase (PTPS, EC 4.6.1.10) and is $Mg^{2+}$and Zn^{2+}-dependent. The final step leading to BH_4 is a succession of side-chain reductions of PTP catalyzed among other enzymes by sepiapterin reductase (SR, EC 1.1.1.153). This reduction is NADPH-dependent.

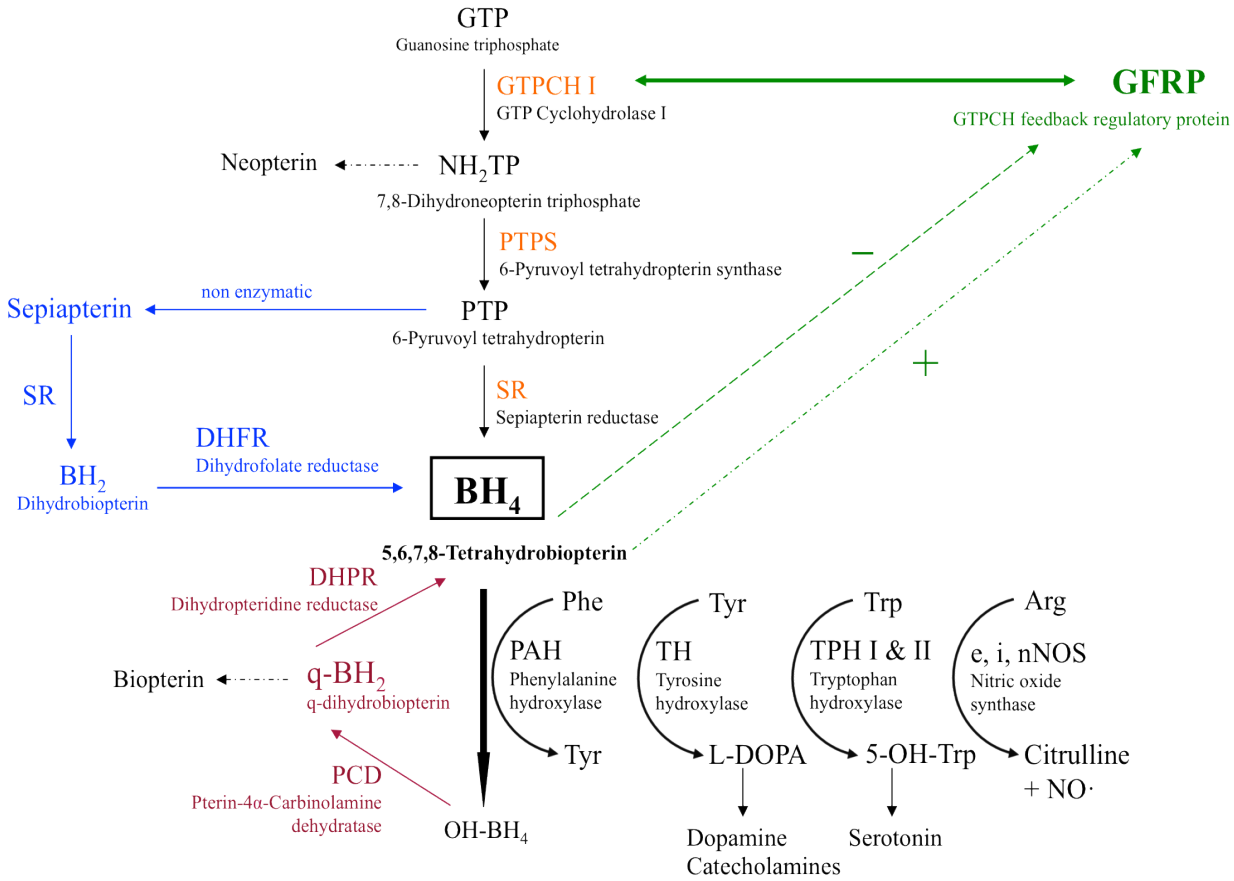


Figure 8: Biosynthesis, regeneration and functions of BH_4. The chemical structures, reactions and regulation details have been extensively reviewed (224, 225).

BH_4 itself is hydroxylated during the aromatic amino acid reactions of PAH, TH and TPH. It is therefore essential that the cofactor be regenerated to ensure a continuous supply of reduced cofactor and to prevent accumulation of harmful metabolites. BH_4 is oxidized to BH_4-4a-carbinolamine and via two further catalytic reactions and quinoid dihydrobiopterin

(qBH_2) intermediate reduced to BH_4. The enzymes involved are PCD and DHPR (red pathway in Figure 8). SR has an alternative function in BH_4 metabolism, namely in the pterin salvage pathway (pathway in blue in Figure 8). This alternative synthesis of BH_4 consists of the conversion of sepiapterin to 7,8-dihydrobiopterin (BH_2) and then via dihydrofolate reductase (DHFR, EC 1.5.1.3) catalysis to the cofactor. Both steps are NADPH-consuming. Sepiapterin is formed from PTP non-enzymatically. PTP can be reduced to BH_4 through several possible routes catalyzed by a family of aldose and carbonyl reductases (AR; CR) via the 6-lactoyl tetrahydropterin intermediate (225, 226).

Intensive research has been performed in BH_4 metabolism and all genes, sequences and structures involved in BH_4 synthesis and regeneration have been described (224). This has greatly contributed in characterization of the genetic disorders in the BH_4 metabolic pathways, which are described in section 1.3.3.

BH_4 acts not only as a cofactor of the aromatic amino acid monooxygenases as described above. Additional functions on the cellular level were found upon the discovery that BH_4 is essential for all three isoforms of nitric oxide synthase (NOS) (227). Nitric oxide synthases convert L-arginine to nitric oxide and L-citrulline (228) in two-step reactions. The role of BH_4 in these reactions is different from aromatic amino acid monooxygenases. BH_4 is not involved in oxygen activation, donates only one electron, and is regenerated without the need for external enzymes. Werner E. et al. recently compared the mechanisms involving BH_4 in hydroxylases and NOSs (225). The synthases are ubiquitously involved in vascular and cardiac functions, establishing a role for BH_4 in diseases like hypertension, diabetes, atherosclerosis, cardiac hypertrophy and failure, but also Parkinson's and Alzheimer's disease (229, 230).

A BH_4-requiring enzyme system for the oxidation of glyceryl ethers was described shortly after the structure of the PAH cofactor had been elucidated (231). However, progress in this field has been limited because the enzyme cannot be produced and purified in sufficient amounts (232). A membrane protein was predicted, named alkylglycerol mono-oxygenase (AGMO, EC 1.14.16.5) and Watschinger et al. (233) recently reported on the sequence assignment of AGMO activity to transmembrane protein 195. AGMO is the only enzyme known to cleave the O-alkyl ether bond in alkylglyercols, using BH_4 to generate an aldehyde and a glycerol derivative and leaving BH_4 in quinoid form. In addition, it is the only enzyme described with a fatty acid hydroxylase motif depending on BH_4 and defining a third group of BH_4-dependent enzymes, next to the aforementioned amino acid hydroxylases and NOSs (225, 233). The physiological significance of AGMO remains to be elucidated.

The many functions and physiological process involvements of BH_4 make the homeostasis of BH_4, bioavailability and regulation highly complex.

1.3.2 Differential Diagnosis of Hyperphenylalaninemias

As already mentioned above, not all individuals with a positive newborn screening result for PKU suffer from a PAH deficiency. An inherited variation in gene coding for one of the enzymes in BH_4 biosynthesis or regeneration pathways also leads to disturbed Phe metabolism and is detected in approximately 1-2% of all HPA patients. The fast response in a BH_4 loading test further pinpoints to BH_4-deficiency and differentiates from non-responsive-HPA/PKU. All neonates with even slightly elevated blood Phe levels need to be screened for the BH_4 deficiencies in order to initiate an early and efficient treatment. The DBS from the Guthrie cards allow the measurement of pterins (neopterin and biopterin), DHPR activity, and amino acids (Phe and Tyr) from a single specimen. The pterin pattern is identical in DBS and urine (234). This leads to the differentiation of all BH_4 defects except SR deficiency. SR deficiency does not present with elevated Phe levels in humans. Biopterin and neopterin are the most important diagnostic metabolites for these disorders. Oxidized pterins are highly blue-fluorescent and can be detected with high specificity and sensitivity after high-performance liquid chromatography (HPLC) in blood, urine, CSF, and amniotic fluid (235). The pterin analysis delivers a characteristic pattern for BH_4 deficiencies, except some patients with DHPR deficiency (234, 236). For example, patients with classical PKU excrete more pterins in urine compared to healthy controls and the amount of excreted metabolite is directly proportional to blood Phe levels (223).

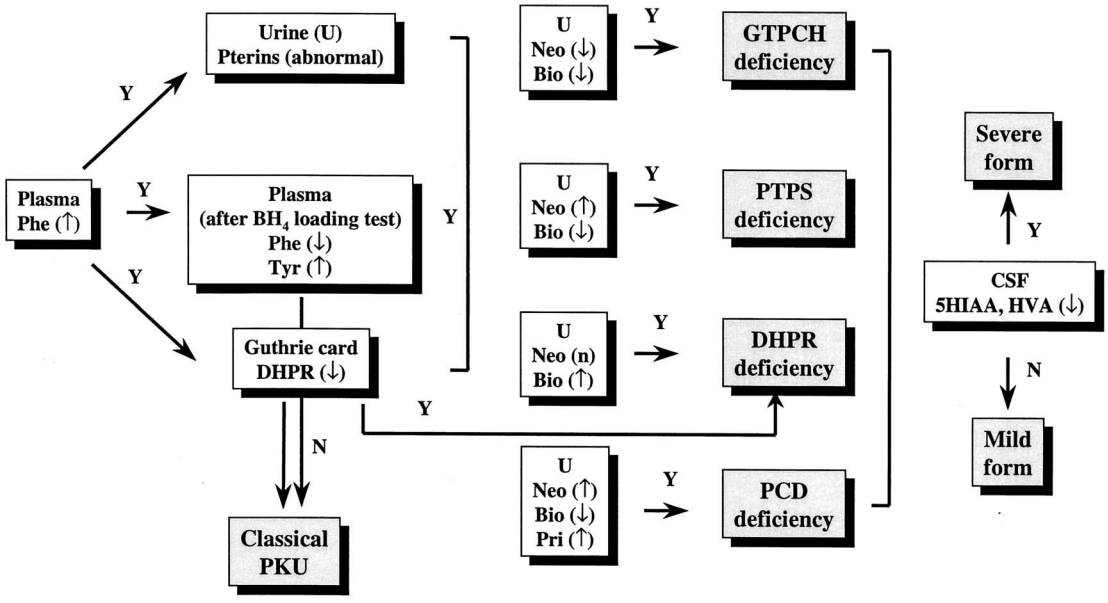


Figure 9: Diagnostic flowchart for differentiation of hyperphenylalaninemia variants (219)

In addition to the pterin measurements, determination of DHPR activity from the blood spots is mandatory in every patient with HPA for differentiation of the underlying defect. The following diagnostic flowchart illustrates the algorithm to follow for diagnosis of some BH_4 deficiencies. An analysis of the biogenic amine neurotransmitter metabolites and folates in CSF adds to the diagnosis of a specific BH_4 disorder and hints to severity.

Table 2 depicts the biochemical markers used in differential diagnosis and discriminates between blood, urine and CSF markers. Whereas the patients with HPA are already detected in neonatal screening programs, those presenting without HPA are recognized either by the typical clinical signs and symptoms or by analysis of neurotransmitter metabolites and pterins in CSF. Further characterization of the disorders can be obtained by measuring GTPCH, SR, PTPS, and DHPR enzyme activities in cultured fibroblasts and by mutation analysis for all variants (237, 238).

Table 2: Inherited disorders of BH_4 metabolism and biochemical markers for differential diagnosis (Table from ref. (225)).

		Blood	Urine			Cerebrospinal Fluid					
Disease	**OMIM #**	**Phe**	**Neo**	**Bio**	**Pri**	**Neo**	**Bio**	**Sep**	**5HIAA**	**HVA**	**Folates**
GTPCH deficiency (autosomal recessive)	233910	↑	↓	↓	n	↓	↓	n	↓	↓	n
PTPS deficiency	261640	↑	↑	↓	n	↑	↓	n	↓	↓	n
PCD deficiency	264070	↑	↑	n - ↓	↑	n	n	n	n	n	n
DHPR deficiency	261630	↑	n	n - ↑	n	n	↑*	n	↓	↓	↓
GTPCH deficiency (autosomal dominant)	128230	n	n	n	n	↓	↓	n	n - ↓	↓	n
SR deficiency	612716	n	n	n	n	n	↑*	↑	↓	↓	n

*7,8-dihydrobiopterin

Abbreviations: ↑: elevated; ↓: lowered; Neo: neopterin; Bio: biopterin; Pri: primapterin (7-biopterin); Sep: sepiapterin; 5HIAA: 5-hydroxyindoleacetic acid; HVA: homovanillic acid; GTPCH: GTP cyclohydrolase I; PTPS: 6-pyruvoyl-tetrahydropterin synthase; PCD: pterin-4a-carbinolamine dehydratase; DHPR: dihydropteridine reductase; SR: sepiapterin reductase.

1.3.3 Inborn errors in Tetrahydrobiopterin Metabolism

BH_4 deficiencies arise from mutations in the genes coding for the enzymes in BH_4 biosynthesis and regeneration pathways. Due to the secondary disturbance in aromatic amino acid hydroxylase metabolism, they also affect central nervous system neurotransmitter biosynthesis and generally lead to severe and heterogeneous phenotypes. BH_4 deficiencies

are treatable diseases although with variable outcomes. The clinical signs and symptoms of a BH_4 deficiency largely arise from neurotransmitter depletion and compromised nitric oxide synthesis (239). The few patients that present with a mild phenotype generally show normal brain neurotransmitter metabolism and require only BH_4 monotherapy. Symptoms of BH_4 deficiency may develop only after a few weeks or months of life. Therefore, most patients are initially maintained on a low-Phe diet until final diagnosis. All BH_4 deficiencies associated with elevated Phe levels are inherited in an autosomal recessive manner and often share common clinical symptoms. Clinical features manifested with GTPCH, PTPS and DHPR deficiencies are abnormal movements together with impaired tone and posture, convulsions, seizures, mental retardation, as well as light pigmentation and microcephaly (16).

Upon deficiency of in GTPCH I, the first step in BH_4 biosynthesis, almost no pterins can be formed and consequently detected in urine or blood. The same accounts for neopterin and biopterin in the CSF and neurotransmitter metabolites are very low (240).

The PTPS deficiency is the most prevalent and heterogeneous form of BH_4 deficiency. Dihydroneopterin triphosphate (NH_2TP) accumulates in tissues of patients, as it cannot be converted to 6-pyruvoyl-tetrahydropterin (PTP). NH_2TP is rapidly dephosphorylated and high amounts of its oxidation product neopterin together with almost no biopterin can be found in patients' samples. In severe forms of PTPS deficiency, patients present with high Phe levels and very low neurotransmitter levels in CSF (219).

In contrast to defects in the BH_4 biosynthesis, higher levels of biopterin are detected in some patients with DHPR deficiency. Some patients with DHPR deficiency can present with completely normal DBS or urinary pterins and DHPR activity measurement is thus mandatory in all HPA patients. Enzymatic activity is completely absent or very low in most reported cases. As BH_4 is not regenerated, feedback inhibition mechanism is disturbed as well. In the absence of DHPR activity, BH_4 cannot be regenerated and 7,8-dihydropterin (BH_2, rearrangement product) accumulates.

Patients with PCD deficiency were initially diagnosed as mildly PTPS-deficient as high neopterin, low biopterin and normal neurotransmitter levels were detected, together with an unknown metabolite in urine. The metabolite was resolved as a 7-isomer of biopterin, namely primapterin, leading to primapterinuria. HPA is only a transient appearance in this condition with only minor clinical abnormalities, such as alterations in tone in the newborn period (219).

Another defect associated with heterozygous mutations in *GCH1*, coding for GTPCH is known as Dopa-responsive dystonia or Segawa disease. Unlike classical GTPCH deficiency,

this disease is not associated with HPA. Mutations are detected only on a single allele of the gene and thus, it is inherited in an autosomal dominant manner with sex-influenced reduced penetrance. Biochemically, neopterin and biopterin levels are reduced in CSF, although not as low as in recessively inherited GTPCH deficiency. Markedly in this disease is a fast clinical response to low doses of L-dopa. Clinical symptoms' onset is in average at age 5 to 6 years. They include muscle dystonia in the extremities and Parkinsonism, hyper- and hypotonia and spasticity later in the age (225, 237).

Characterization of defects in SR was achieved only twelve years ago (241). Diagnosis of patients with this disease was probably missed because of lack of HPA and normal pterin excretion in blood or urine. Biochemical features are rather similar to DHPR deficiency and compensation is possible due to the peripheral salvage pathway to produce BH_4 in the liver. The biochemical abnormalities can only be found in CSF with low levels of homovanillic and 5-hydroxyindolacetic acids, as well as high levels of total biopterin, BH_2, and sepiapterin. Clinical features of SR deficiency include microcephaly, dystonia, progressive psychomotor retardation and extrapyramidal symptoms (219, 242).

In the non-hyperphenylalaninemic BH_4 deficiencies, the production of the cofactor in peripheral tissues is sufficient for normal PAH activity. However in a Phe-loading test, it can be observed that tyrosine production is compromised (219).

Due to the similar features among the BH_4 disorders, therapies may also be similar, generally including supplementation of L-dopa/carbidopa (peripheral decarboxylase inhibitor) as well as 5-hydroxytryptophan. BH_4 serves as well as a treatment component, depending on the variant. Clinical and biochemical outcomes vary with age of diagnosis, so that a careful follow-up and fine-tuning of treatment is required (154).

Two online resources are available at the www.biopku.org website, BIODEF and BIOMDB databases, which catalogue clinical, biochemical and molecular data of almost 700 patients with BH_4 deficiencies. Information is also given about severity, phenotype, and origin. BIOMDB is linked to BIODEF and lists mutations causing BH_4 deficiencies and some other pediatric neurotransmitter diseases.

1.4 *In Vitro* Expression of PAH Mutations

The three major approaches have proven very fruitful in exploring the molecular causes of PKU: 1) Patient-based investigations analyze the effect of mutations on enzyme function in human organism. Many aspects from mutation detection, population studies, and clinical data are combined for the phenotype definition. 2) Three-dimensional protein structural

studies consider the examination of the isolated recombinant mutant enzyme. 3) Analysis of mutation effects on enzyme activities by *in vitro* functional assays links the patient data with the protein. This is probably the closest available *in vitro* approximation to the *in vivo* hepatic PAH, which is not readily available (243).

The main goals of *in vitro* expression (IVE) and co-expression studies are: 1) the confirmation of a disease-associated mutation and its pathology; 2) the severity of a mutation's impact and predicting the corresponding metabolic phenotype; and 3) the understanding on how a mutation exerts its deleterious effects (study of molecular mechanisms). In principle, IVE analysis involves the introduction of the human wild type *PAH* cDNA into a plasmid vector, then its introduction into host cells lacking endogenous PAH, and the characterization of resultant enzyme. PAH missense mutations can be introduced by site-directed mutagenesis into the plasmids for the study of mutations' effect on protein function. The report demonstrating that the recombinant gene product preserves most of the biological characteristics of the native protein, and the facilitation of producing greater amounts of high purity of recombinant hPAH variants, promoted tremendous advancements in characterizing PAH deficiency (183, 244). Depending on the aims and downstream analyses of the study, several expression systems have been applied to study *PAH* mutations. There is no perfect single expression system, but many mutations have been studied in different systems and only combined results give useful information on their effects on enzyme function.

The expression of PAH in bacterial systems like *Escherichia coli* has been intensively employed. PAH is generally expressed as a fusion protein that is less susceptible to proteolytic degradation. The high amounts of pure PAH protein allows detailed enzymological studies in terms of kinetics, stability, and oligomerization. In addition, they created the source for the crystallization and modeling of the PAH structural details. These models greatly help in analyzing structural implications of mutations. A drawback of high amounts of protein expression in bacteria is their tendency for aggregation. This requires further analysis of the isolated dimeric and tetrameric forms of PAH to distinguish them from such bacterial artifacts. Unfortunately, although seemingly identical expression systems are used, different labs observed different catalytic, physical and regulatory properties of the wild type PAH. These can probably be explained by experimental discrepancies.

Oligomeric structures of wild type and mutant PAH enzymes were evaluated in a cell-free *in vitro* transcription-translation system (245, 246). Rabbit reticulocyte lysates are used as host instead of intact cells. These provide a mammalian cell milieu containing the necessary

cellular components for protein synthesis. An RNA polymerase specific for the promoter in the expression system is added to allow high levels of transcription from the cDNA. This is directly coupled to translation and inserts labeled methionine into the nascent protein for rapid quantification. Experimental additions of cofactors, inhibitors or other compounds are facilitated. Enzyme activity as well as time-course experiments can be performed with these lysates (216, 247, 248). However, the levels of PAH protein produced are rather low and limit broader applications of this system. Gjetting et al. (249) mentioned inconsistent correlation of results determined with an *in vitro* transcription-translation system and a mammalian system. They concluded that differences in the environments of the two systems, including phosphorylation and control protein quality, might affect some mutant forms more than others.

Transient overexpression of PAH cDNA integrated into a plasmid vector in mammalian cells was the first type of system used to analyze mutant forms of PAH. It is nowadays still the closest approximation to the *in vivo* milieu and first-line approach to studying disease-causing effects of mutations. The host cells are generally COS (monkey kidney) or Hek293 (human embryonic kidney) cells, because they do not possess endogenous PAH activity, but otherwise resemble the hepatic and renal cells in which PAH is naturally expressed. PAH activity measurements in protein lysates reveal differences in activity for the various mutants expressed. Analysis of immunoreactive PAH protein, as well as quantitation of mRNA compare mutant and wild type expression levels. The expression level of a mutation compared to wild type PAH indicates whether the main effect of a mutation is only on residual activity or also on reduced protein amounts. Therefore, mammalian expression systems require a certain number of controls for example for transfection efficiency, endogenous amino acids and eventual normalization of mRNA levels.

All of the above described expression systems lead to the production of homomeric forms of PAH protein, in which all subunits are identical. As most PKU patients are compound heterozygotes with each *PAH* allele carrying a different mutation, two-hybrid analysis is applied. This methodology allows the semi-quantitative assessment of protein-protein interactions occurring in living cells. Co-expression systems were reported with bacteria, yeast, and mammalian cells as host (250, 251, 252). These systems allow the study of disease-causing mutations upon normal assembly of the tetrameric PAH enzyme. The PAH monomers are expressed as fusion proteins; one with a DNA-binding domain (BD) and the other with a transcriptional activator domain (AD). Interactions between PAH monomers bring the two domains into close proximity, leading to activation of reporter genes. Reporter

activities are decreased compared to wild type upon influence of mutations on normal interactions. However, two-hybrid systems are not the best available milieu for studying PAH enzyme activities (250).

In many cases the different expression systems complement each other. For example, mammalian systems fail to explain the instability of mutant proteins, concluded from lower levels of mutant protein generally observed compared to wild type expression. But, for example the question of proteolytic degradation was successfully addressed in bacterial systems (253). In conclusion, *in vitro* expression studies have greatly contributed to today's knowledge of PAH deficiency and understanding of molecular mechanisms involved.

1.5 PAH Misfolding as Cause of PKU

Mutations that affect the stability of a protein's 3D structure or interfere with the folding process are commonly found in many inherited diseases. These effects can lead to increased aggregation and destabilized native conformation, causing loss-of-function pathologies (or eventually gain-of-function). These aggregates or nonfunctional conformations are no longer removed by the cells quality control systems like proteases. Replacements in amino acid sequence can abolish native interactions between atoms or create novel non-native interactions, changing the topology of the energy landscape and lead to misfolded conformations (254). Many of the point mutations on the *PAH* sequence leading to missense in translation were found to affect protein folding and assembly leading to secondary effects on enzyme function. The protein architecture of PAH is very sensitive to single point mutations throughout the protein sequence. The successful cloning of *PAH* cDNA and isolation of recombinantly expressed PAH allowed thorough studies of biochemical and physicochemical properties of wild type and mutant PAH proteins (12, 183, 244). The molecular mechanisms of many mutations causing PAH deficiency were analyzed either in *in vitro* expression studies or through three-dimensional protein structural studies. In summary, these studies demonstrated decreased stability, disturbed oligomerization, formation of aggregates and accelerated degradation of variant PAH proteins (246, 249, 255, 256, 257). All these processes are likely to be mutually dependent and their combined actions lead to impairment of the phenylalanine hydroxylase system. As there is still a general lack of knowledge in protein folding, it is not yet predicable whether a variant leads to misfolding of the protein or not (258). The results obtained from analysis of many *PAH* mutations in IVE systems are compatible with a unifying model. This model is shown schematically in Figure 10 and depicts the various pathways in protein folding. Any newly-synthesized polypeptide has the potential to be partitioned between correct and aberrant pathways for folding and

oligomeric assembly (259). This is because the monomeric folding intermediate is in equilibrium with one or more misfolding intermediates.

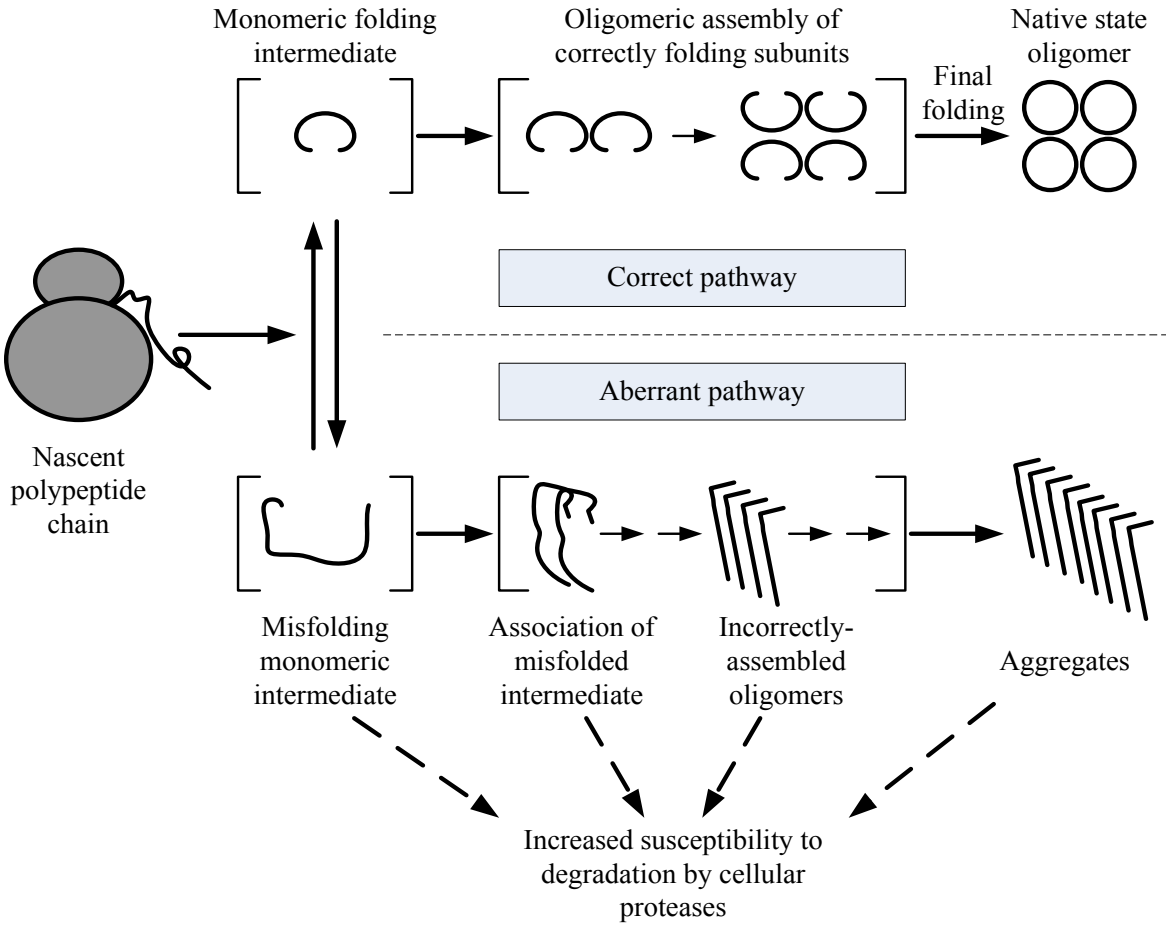


Figure 10: Model of competing pathways for correct versus aberrant protein folding and assembly. (Figure based on ref. (260))

Mutations and/or environmental factors can adversely affect rates of correct monomer folding, or can oppose the correct assembly of monomers into native-state oligomers. In either case, this will result in a greater proportion of protein entering the aberrant pathway, where it is more susceptible to proteolytic degradation in human cells than is protein proceeding through the pathway of correct folding and assembly (260). Significant efforts were made in recent years to establish a consistent relationship between the clinical phenotype, *PAH* genotype and *in vitro* residual enzyme activity. The view on global conformation of PAH was clarified in a more recent study by Pey et al. (261) using the algorithm resource FoldX and predicting the effects of missense mutations on stability. They confirmed that the decrease in protein stability is the main molecular pathogenic mechanism in PKU and the determinant for phenotypic outcome. In addition, they demonstrated substantial overall correlations between the mutational energetic impact and both, *in vitro* residual activities of 80 mutations in eukaryote systems and patient metabolic phenotype

(261). Additional results were also reported by Gersting et al. (262) who studied the molecular basis of structural mechanisms leading to loss-of-function in PKU by recombinant mutant analysis. Their findings are well in agreement with the computational analyses by Pey et al. (261). These studies emphasize that mutations impair catalysis, but also aberrant folding and conformational destabilization at different degrees are causes of PAH deficiency. The mutations were shown to impair molecular motion needed for regulatory processes such as substrate and cofactor binding, activation and inhibition, as well as promoting changes in the oligomeric state. The PAH enzyme should be regarded as an entity of functional units with crucial networks of amino acid interactions being disrupted in the tertiary and quaternary protein structure and not only disturbance of the three domains of the primary structure (262).

These structural data confirm that molecular chaperones, like BH_4, can be an efficient treatment option for PKU. Molecular chaperones assist the folding by protecting the protein during the folding process and keeping it away from misfolding which may lead to aggregation. The targets for chaperones are unfolded and partially folded polypeptide chains with exposed stretches of hydrophobic amino acids that are usually sequestered in the core of folded proteins (258).

1.6 Splicing Defects in PKU

RNA splicing is the process of removing introns in a typical mammalian gene for the subsequent precise joining of exons. In mammalian cells, the production of several isoforms from the same transcription unit by various types is a common event known as alternative splicing. Alternative splicing is responsible for much of the complexity of the proteome. It leads to the expression of different variants in different tissues, in different cells within the same tissue, or in the same tissue at different stages of development or in response to pathological processes. The understanding of the biological function and abundance of splice variants contributes to elucidating alterations in disease states. It is estimated that 94% of human genes are alternatively spliced and that as many as 50% of disease-causing mutations affect splicing (263, 264). Mutations that affect splicing can cause disease directly or contribute to the susceptibility or severity of disease. For example, the disease mechanisms of cystic fibrosis, spinal muscular atrophy and medium chain acyl dehydrogenase complex have been extensively studied and determined as splicing defects (265).

Splicing is carried out by spliceosomes, which are ribonucleoprotein complexes that recognize the exon-intron junctions and catalyze the precise removal of the introns. The

splicing process and alternative splicing can be disrupted both by mutations within the target gene sequence (*cis*-acting elements) required for correct pre-mRNA processing as well as by mutations that affect *trans*-acting components necessary for splicing regulation. The *cis*-acting elements are small pre-mRNA sequences recognized by the spliceosome and they are referred to as either intronic or exonic splicing enhancers (ISE or ESE) or splicing silencers (ISS or ESS). The *trans*-elements are specialized protein families that either positively or negatively regulate exon recognition and inclusion in the product. A critical balance between these antagonistic regulators is necessary for controlling the level of exon inclusion in the mRNA transcript (266).

The cloning of the PAH cDNA led to the identification of PAH mutations leading to aberrant mRNA processing and deletions. Before the era of polymerase chain reaction (PCR) techniques and sequencing of all 13 exons of PAH, population studies using RFLP haplotype characterization of the PAH locus were efficient in defining a few splicing mutations in common haplotypes. The first PKU mutation identified was a single base substitution in the canonical 5'-splice donor site of intron 12, known today as IVS12+1G>A (c.1315+1G>A) (267, 268). At present, the McGill PAH database lists 65 splicing mutations in almost every region of *PAH*, leading to insertions and deletions. Splice mutations are generally point mutations occurring in introns and affecting the classical consensus splice site signals. They are often located close to the exon-intron boundaries, namely in the 5'- and 3'- splice regions of each exon. These sites are particularly important for exon recognition in the splicing process, so that these mutations deleterious effects in the gene processing are the consequence. Four out of the 65 splice mutations are located within one of the PAH exons. Particularly, the three exonic PAH splicing mutations in the database are even silent mutations resulting in PKU phenotypes. Silent mutations may act on mRNA level and are probably under-reported, because they might be incorrectly assumed to be neutral polymorphisms that do not merit further characterization.

The discovery of illegitimate transcription greatly facilitated the detection of *PAH* mutations and of allelic variants leading to alternative gene transcripts (269). This phenomenon explores the low transcription levels of tissue-specific genes in non-specific cells. Circulating lymphocytes give easy access to *PAH* gene transcripts with identical sequence to that reported in liver. The transcripts are amplified by PCR, reverse-transcribed and sequenced (270, 271). The transformation of lymphocytes with Epstein-Barr virus (EBV) creates an immortalized cell line, 'frozen' at the stage in differentiation achieved when the transformation occurred (272). Such cell lines can be used for extensive molecular genetic

studies permitting analysis of cDNA as well as genomic DNA, possibly revealing mutations buried deep in introns not detected by other commonly used methods.

As can be seen in the following examples, exonic PAH mutations were also shown to affect the splicing process. The study of the p.Y204C mutation represents a strong example of the importance of mRNA processing studies. The mutation was detected in patients with classical PKU phenotype, but *in vitro* residual enzyme activities and immunoreactivities did not correlate with the severe damage found in patients. Analysis of mRNA from cultured lymphocytes by RT-PCR revealed a 96 base pair deletion in exon 6 and the generation of a novel 5' donor splice site. The mutation was thereafter renamed to Ex6-96A>G (273).

Similarly designed studies using cultured lymphocytes as a source for PAH transcript analysis were employed to explain discrepancies in correlating *in vitro* expression data with patients phenotypes in nonsense, p.G272X and p.Y356X, and missense, p.P281L and p.R408Q, mutations (274).

In addition to the lymphoblast analysis, transient expression of minigenes is used in *in vivo* assays for further assessment of a mutation's effect on splicing mechanism (275). The c.1197A>T silent mutation (p.V399V) proved pathogenic and results in a PKU phenotype by inducing PAH exon 11 skipping and resulting in an altered reading frame. The mutation leads to an unfavorable situation in exon recognition and stresses the influence of splice donor and acceptor sequence conservation (276) as well as the probability of affecting ESEs. It is now re-classified as disease-causing mutation.

The consequences of a synonymous mutation influencing splicing signaling sites is demonstrated by Dobrowolski et al. (277). An *in vivo* minigene approach is used together with the analysis of splicing proteins, so-called *trans*-factors, to identify splicing regulation upon c.30C>G neutral variation. Several lines of evidence are presented that this benign change is a pathological mutation. The c.30C>G mutation is shown to create an exonic splicing silencer site and interacts with the hnRNPH, known as a splicing inhibitory protein and leads to disruption of mRNA processing and aberrant protein production. The authors state that such observations are relevant for interpretation of other PAH exonic mutations and implications need to be considered for potential response to BH_4 therapy.

However, these are not the only examples and therefore it should not be generally assumed that full-length transcripts are produced from silent and especially also missense mutations (260, 278, 279, 280). This effect has long gone unnoticed in mutation analysis. The *PAH* gene has over 400 recognized exonic mutations and it is with certainty that a significant portion of these will realize their molecular pathology by disrupting mRNA splicing despite

the fact that they ostensibly create missense, nonsense, single codon deletions, or neutral changes according to the genetic code (277, 281, 282). This phenomenon may add another complication in genotype-phenotype correlations, resulting from unexpected effects of mutations in the coding region on the splicing process. The effects of nonsense, missense and silent mutations should be more routinely evaluated to assess their possible consequences on pre-mRNA processing. The identification of abnormal splicing as the primary mechanism of disease raises the possibility of therapeutic approaches that target splicing.

1.7 Biochemical and Molecular Mechanisms of Tetrahydrobiopterin Responsiveness

Since the initial observation of decreasing Phe levels after oral intake of BH_4 by Kure et al. (150), an increasing number of patients have been reported. BH_4 responsiveness is defined as the response to 10 - 20 mg/kg body weight of the natural cofactor BH_4 with a reduction of more than 30% of plasma Phe levels during the first 8 - 24 hours after administration. Of course, BH_4-deficiencies in cofactor synthesis or recycling must be excluded. Kure et al. initially proposed that PAH mutants are formed exhibiting reduced binding affinity for BH_4. The increase in BH_4 upon supplementation would lead to the restoration of the residual activity and is likely to stabilize mutant PAH proteins. Many reports followed presenting similar efficiency of BH_4 treatment in patients with mild and moderate phenotypes, often compound heterozygote with one mild mutation (151). It was emphasized from these studies that a significant residual PAH activity is a prerequisite for BH_4 responsiveness. This was further characterized on biochemical, molecular and physiological levels and several possible mechanisms for BH_4 responsiveness were proposed based upon results of *in vitro* expression studies and structural implications of the mutations (283). Many mutations and genotypes were found associated with responsiveness and are listed in the BIOPKU database (www.biopku.org).

Initially, it was thought that mutations in the catalytic domain, especially residing in one of the four cofactor-binding regions (CBR) or in locations that directly interact with these regions, would be responsive to BH_4. This correlated with the BH_4-responsive mutations found in previous patient studies. The cofactor binding regions are depicted in Figure 11 highlighted in dark blue. The cofactor binding regions include amino acid residues 246 to 266, 280 to 283, 322 to 326 and 377 to 379 (283).

The mutations have been analyzed in *in vitro* kinetic studies on recombinantly expressed mutant PAH and compared to wild type data (216, 285, 286, 287). Several of the mutations

were found to exhibit lower binding affinities towards BH_4 compared to the wild type (Table 3).

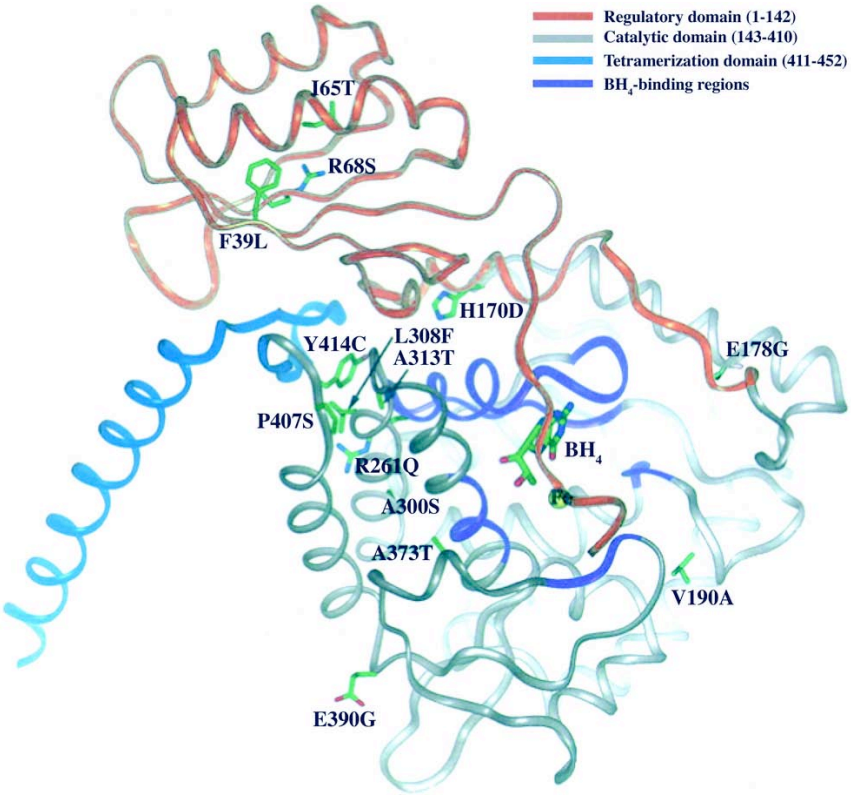


Figure 11: Backbone structure of a monomer of the composite model of PAH (Figure from ref. (284))

Table 3: Binding affinities for BH_4 in mutant PAH fusion proteins expressed in *E.coli* (K_m or K_d defects). Data compiled from (216, 285, 287)

PAH proteins	K_m[a] (BH_4) µM	K_d[b] (BH_4) µM
Wild type	26 ± 3	2.7 ± 0.1
p.F39L	44 ± 2	8.4 ± 0.8
p.I65T	39 ± 3	3.9 ± 0.4
p.R68S	30 ± 3	9.0 ± 1.0
p.D129G	37 ± 3	ND
p.P244L	39 ± 4	ND
p.R261Q	25 ± 2	2.7 ± 0.1
p.L308F	44 ± 8	ND
p.A309V	38 ± 3	ND
p.V388M	24 ± 3	ND

a, Obtained by steady-state kinetic analysis
b, Obtained from equilibrium binding measurements by isothermal titration calorimetry
ND, not determined

This is represented in higher values of the Michaelis-Menten constant K_m, which is a measure of the binding affinity of an enzyme for its ligand (substrate or cofactor) and is defined as the concentration of ligand required to fill one-half of ligand-binding sites (217) in steady-state kinetics. The dissociation constant K_d is also a measure of affinity for a given ligand to a protein. For most of the mutations displayed inTable 3, K_d is elevated compared to the wild type, representing reduced affinities of mutant PAH and BH_4. The BH_4-responsive mutations in Table 3 proven to be K_m mutants (p.F39L, p.I65T, p.R68S and p.D129G), are rather located in the regulatory domain of the PAH protein than in the cofactor binding regions. In addition, the p.D143G mutation was rigorously studied in multiple systems and significant increases in K_m were found, concluding a pure kinetic variant (288). As the initial hypothesis of only K_m mutants being BH_4-responsive does not hold for all the currently characterized mutants, other effects must play a role and another mechanism emerged, based on the results from *in vitro* studies. Kure et al. later suggested from results obtained in a PKU mouse study, that responsiveness to BH_4 was probably due to suboptimal physiological concentrations of the cofactor in hepatocytes (289). A hepatic concentration of BH_4 of 5 - 10 µM has been measured. However, as Michaelis constant varies between 12 and 44 µM for wild type PAH, the enzyme is not saturated at this concentration of cofactor (285).

Pey et al. (207) showed that PAH activity, in physiological concentrations of Phe, is almost not sensitive to the concentration of BH_4 when varying from physiological to superphysiological levels (Figure 12).

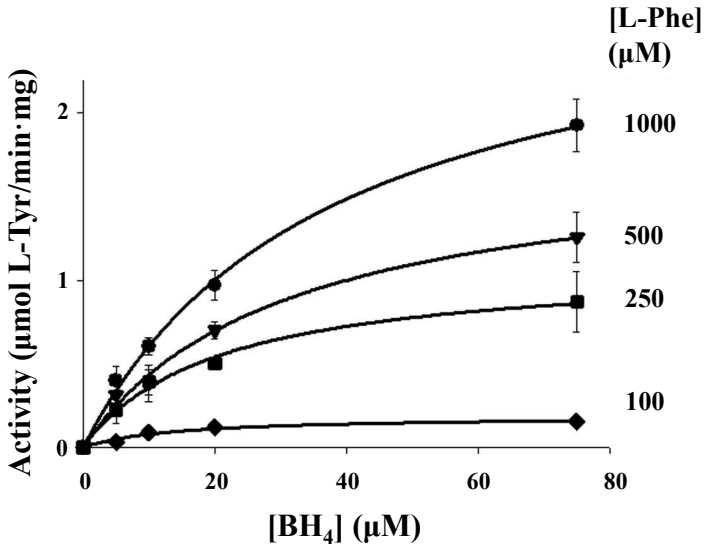


Figure 12: PAH activity dependence on the concentration of BH_4 at different concentrations of L-Phe (Figure adapted from ref. (207))

However, this was shown to change at increased pathological Phe concentrations, mainly due to a 5-fold increase in K_m (BH_4). This remarkable regulation of wild type activity by Phe and BH_4 is highly susceptible and easily disturbed by conformational changes provoked by mutations (207). PAH is known to undergo conformational changes upon Phe binding leading to activation, whereas BH_4 is a negative regulator, leading to an inactive PAH-BH_4 complex. However, Phe binding can surpass this closed conformation and lead to increased conversion of Phe to Tyr. In this sense, BH_4 loading would prevent the mutant enzyme from structural changes caused by mutations and prevent from misfolding and proteolytic degradation. A reduction or loss of the cooperative substrate-dependent activation has been seen with many mutations kinetically characterized. This stabilizing effect of BH_4 on PAH mutants was determined by thermal denaturation studies with increased melting temperatures and half-lives for the mutant proteins in the presence of the cofactor. In the absence of the cofactor, mutant proteins show increased rates of degradation, indicating a folding defect (248). The cofactor may prevent wild type and mutant PAH from degradation by the ubiquitin-proteasome-dependent pathway (253). These observations attribute BH_4 a chaperone-like protective effect for PAH by stabilizing the functional forms. This hypothesis is supported by an increase in enzyme activity and protein amount with BH_4 content *in vivo* in mice (290). However, BH_4 does not have an effect on PAH gene transcription or mRNA stability (291, 292). In addition, a chaperone effect of BH_4 also explains highest efficiency with milder phenotypes, as sufficient residual liver PAH protein must be present to interact with the cofactor.

The establishment of activity landscapes by Pey et al. (207) and Staudigl et al. (293) of PAH wild type and mutants supports the influence of kinetics in response to the cofactor. They allow the analysis of a wide range of substrate and cofactor concentrations, also relevant for diagnosis and treatment of patients with PAH deficiency. This highlights the interplay of both kinetic and chaperone effects varying with the genotype and influencing the therapeutic effect. However, the interindividual differences in pharmacokinetic properties can still lead to different degrees of responsiveness associated with identical genotypes. In conclusion, the response to BH_4 therapy is multifactorial, and can be explained by a combination of proposed mechanisms attributing correction of kinetic and stability defects to the favorable action of BH_4. Continuous characterization of PAH mutations and BH_4 responsiveness testing leads to the identification of new BH_4-responsive mutations and the improvement of current models for the molecular basis of cofactor-responsive PKU.

1.8 Genotype-Phenotype Correlations

The compilation of all information about *PAH* mutations and establishment of genotype-phenotype correlations is highly useful in predicting the course of disease for a patient's diagnosis. The ability to predict the phenotype already in a newborn with PAH deficiency not only enables the design and early implementation of an optimal dietary regimen, it also greatly improves counseling of the patient's family. However, contrary to initial beliefs, a simple correlation between *PAH* genotype and metabolic phenotype could not be confirmed.

The enormous allelic heterogeneity in PAH deficiency with almost 600 known mutations seems to be a severe constraint for attempts to establish genotype-phenotype correlations. The number of mutations has doubled over the past decade. The genotype is not always the best predictor for the clinical phenotype (58) and many inconsistencies were reported in correlating genotype with phenotype. Nevertheless, it was also possible to document clear correlations between the allelic variants and the metabolic phenotype in some studies (294, 295). Despite of the many factors influencing phenotypes, the specific *PAH* genotype is the main determinant of metabolic phenotype in most cases (40, 42).

Differing clinical phenotypes were reported in some cases with identical genotypes, even in siblings. Mechanisms leading to such interindividual variations in Phe kinetics and brain Phe content are still unclear and under investigation. Moreover, as seen above, the usage of a different IVE system may lead to different conclusions of a mutation's impact and even the results from similar systems are sometimes difficult to compare (249). The cellular environment in IVE experiments is an important component in terms of folding, oligomer assembly, and aggregation of the mutant enzyme. Therefore varying patient phenotype data upon identical genotypes need to be included in databases and considered when establishing correlations with a mutation's outcome. However, as there exists no consensus in classification of phenotypes and Phe tolerance, alleged genotype-phenotype inconsistencies can be a result of phenotype misclassification. In a large multicenter study, inconsistencies were attributed to the various phenotype categories among countries, various genotyping methods between centers as well as different treatment guidelines (42). The moderate phenotype category, relating to Phe levels between 900 and 1200 µmol/L, is not routinely considered by every center. However, most phenotypes not correlating to genotypes belong to this category, which adds additional confusion in assigning a patient's phenotype.

In vivo residual PAH activities are often calculated based on averaging *in vitro* expression activity data. The activity of both alleles in percent of wild type is averaged to account for the full genotype. Some variability in the data is not inevitable when different expression

systems are used and these values tend to overestimate the *in vivo* situation (252). In addition, predicted residual activities do not account for interactions between the two alleles influencing enzyme activity, so that dominant effects of one of the alleles may lead to a different phenotype than expected from prediction. Negative interallelic complementation has been demonstrated for a few genotypes, but heteromeric enzyme activities are difficult to determine (250, 296). Thus, they allow ranking a mutation in mild, moderate or severe phenotype. Combining predicted residual activities with patient data might lead to useful correlations, but one has to be aware of the limitations of IVE systems, that might fail in reflecting the complete *in vivo* situation. In patients with new or rare mutations, little knowledge is available and IVE thus leads to fast informative data.

A few common PAH mutations have been repeatedly reported in inconsistent genotype-phenotype correlations. These mutations were mostly classified with more than one phenotype category and include: p.L48S, p.I65T, p.R158Q, p.R261Q, p.V388M, p.E390G, and p.Y414C. When differences in classification between participating centers were excluded, explanations were ascribed to the position of the mutations. In detailed studies of these mutations, the position within the protein was found to influence activity because of interference with binding of the substrate or the cofactor. This hinders efficient cooperativity or leads to severe folding defects (255, 285). The co-overexpression with chaperonins GroES and GroEL (247, 255, 297) revealed different results for activity and oligomerization patterns in some of these mutant proteins. Increased levels of protein and residual activity with chaperonin overexpression rather point to folding defects. In addition, the influence of Phe levels on activity modulated conformational changes happening typically after substrate binding in PAH (298). Enzyme activity may therfore be subject to regulation by substrate concentration. The recording of an activity landscape for the p.R261Q mutation showed that Phe levels greatly influence residual activity and BH_4-response in this mutant (293). In these cases, standard PAH activity protocols would not efficiently reflect the *in vivo* situation, as only one substrate and cofactor concentration is applied.

These examples prove that not only the implications of a mutation on activity and structure, but also the levels of substrate and cofactor need to be considered in genotype-phenotype correlations. The combination of all these effects might lead to more consistent correlations and confirms the need for continuing *in vitro* expression studies and further characterization of PAH mutations.

1.9 Objectives of the Thesis

PKU has been listed in the McKusick *Catalog of Mendelian Inheritance in Man* (OMIM, # 261600) since 1986 as a monogenic disorder with known straightforward pattern of inheritance. The OMIM compendium focuses on the relationship between phenotype and genotype, adding value to prognosis and treatment. However, the preceding subchapters have illustrated that PKU is not a simple trait but it is a highly heterogeneous disorder with a broad spectrum of biochemical and metabolic phenotypes. Figure 13 summarizes the factors adding to the complexity of monogenic PKU resulting in challenging genotype-phenotype correlations.

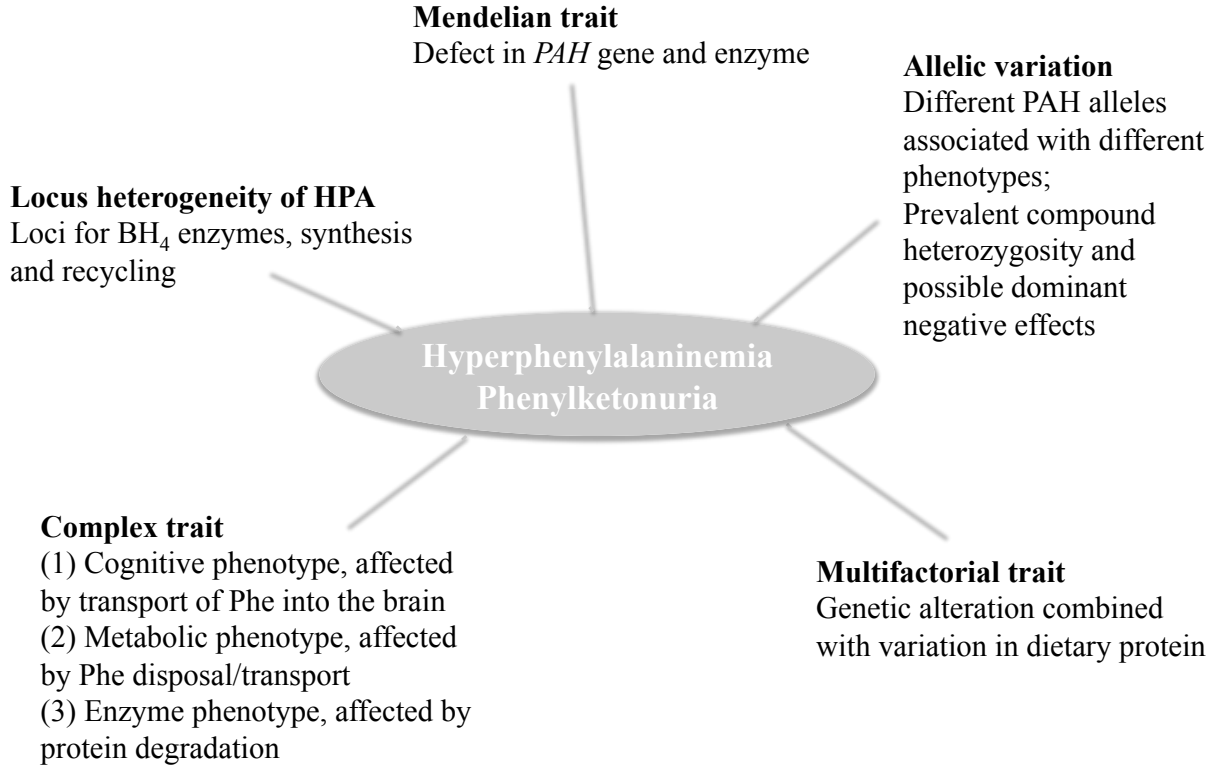


Figure 13: Factors influencing phenotype in phenylketonuria (Figure adapted from (299))

The prediction of the 'monogenic' phenotype from the *PAH* genotype is severely challenged by the fact that patients, even siblings, with identical *PAH* genotypes could have greatly different cognitive and metabolic phenotypes. Most probably, this results from other genetic contributions to the phenotype (e.g., modifier genes), which are complex and sparsely investigated in PKU. Many of the features mentioned in Figure 13 also greatly influence the prediction of BH_4 responsiveness and often lead to inconsistent results. But as 20 - 30% of PKU patients are known to respond to oral supplementation of BH_4 and can therefore benefit

from this pharmacological therapy, information on genotype, potential residual activity, and likely Phe tolerance are relevant for therapeutic approaches.

The aim of this work was the metabolic characterization of genotypes associated with BH_4 responsiveness. The thesis project outlines the molecular, *in vitro* analysis of several common, but also new PKU mutations and testing of their response to BH_4 in a mammalian cell system for better prediction of BH_4 responsiveness.

In *Chapter 1*, we aimed at characterizing the diversity of genotypes in Turkish PKU population. Genotyping of patients with elevated Phe levels detected in newborn screening is often performed to complete diagnosis of a PKU patient. A higher prevalence of PKU is found in Turkey with 1 in 6'500 newborns. Only two studies reported on mutations prevalent in Turkey. Blood and urine samples from many of the patients selected for genotyping were previously registered in our division and a BH_4 loading test was performed. Phe levels as well as information on BH_4 formulation used during the loading test were therefore available. From these data, we planned to correlate genotypes with response to BH_4-loading, phenotypes, and predicted residual activities from *in vitro* expression tabulated in *PAH*db. The results will be added to the BIOPKU database, which is public and new patients with documented non-responsive genotype may not need to undergo BH_4 testing.

The aim of the patient study in *Chapter 2* was to determine the predictive value of BH_4-responsive PAH mutations in Croatian PKU population. The prediction facilitates the selection of potential PKU candidates for pharmacological therapy with BH_4. Null mutations for example are generally not associated with response to BH_4. Many inconsistent results have been reported on single PAH mutations in relation to BH_4 responsiveness in milder phenotypes. It was envisaged to select patients according to substantial residual activity of one allele and evaluate BH_4-response after the loading test.

Chapter 3 describes the quantification of PAH activities expressed in cultured cells or in animal tissues by a novel tandem mass spectrometry assay. The measurement of PAH activities was usually achieved by the amino acid analyzer. However, the amino acid analyzer was found not sensitive enough to quantify Phe and Tyr in cell lysates, especially in cells expressing mutant PAH with low activity. Mass spectrometry allows the use of stable isotopes for amino acid quantification with increased specificity. In addition, we intended the validation of this analytical method. Validated PAH assays have not been thoroughly reported and comparison of mutant PAH activity between various assay methods is difficult. With tandem mass spectrometry, we aimed at using new technology for amino acid

quantification with a broad concentration range allowing the accurate determination of PAH activity.

Furthermore, several particular mutations found in Turkish PKU patients were selected for detailed molecular analysis illustrated in *Chapter 4*. It is generally underestimated, how many missense mutations influence splicing mechanism and lead to aberrant splicing. Mutations in exonic splicing enhancers and silencers, as well as exon splice sites greatly contribute to efficiency of exon recognition by the spliceosome. The impact of such mutations may be more severe than the effect predicted from a missense change on enzyme function. In case of aberrant splicing, residual activity does not correlate with phenotype and this may be causing frequent inconsistent genotype-phenotype correlations. Mutations that disrupt splicing are unlikely to facilitate response to BH_4 and if not recognized, their effect on splicing may lead to further inconsistent genotype-phenotype correlations.

Moreover, the most prevalent PKU mutation, c.1066-11G>A is an intronic mutation causing aberrant *PAH* splicing. Our intent was to investigate the association of a mutation in *PAH* intron 10 with BH_4 responsiveness and two mutations in *PAH* exon 11. Discordant PAH activity results were found for these mutations, as well as their location in important splicing regulatory elements pointed to an implication on *PAH* exon 11 mRNA processing. The establishment and analysis of *PAH* minigene constructs and EBV-transformed PKU patient cell lines were planned for investigation of these mutations in the 3' splice site and in exon 11.

The final section, *Chapter 5*, presents the setup and validation of a mammalian cell test system with engineered plasmid vectors. The previous studies have shown that the combination of the two mutant PAH alleles determines BH_4 responsiveness *in vivo*, and the individual mutations of a patient should not be viewed by themselves. Transient co-expression of wild type or mutant PAH variants of previously selected genotypes and measurement of PAH activity and expression levels, in presence or absence of exogenous BH_4 will be tested. A robust method for assessing responsiveness in cell culture and prediction of BH_4-response of a specific genotype is fast, cheap and prevents some patients to undergo the burden of BH_4 loading test. In addition, we expect to establish dominant influences of some mutations on the enzyme function. The analysis of numerous mutation combinations by the same system will enable the establishment of algorithms for the calculation of genotype-based BH_4 responsiveness.

1.10 References

[1] Følling, A. (1934). "Über Ausscheidung von Phenylbrenztraubensäure in den Harn als Stoffwechselanomalie in Verbindung mit Imbezillität." Hoppe-Seylers Z. Physiol. Chem. 277: 169 - 176.

[2] Følling, A. and Closs, K. (1938). "Über das Vorkommen von l-Phenylalanin in Harn und Blut bei Imbecillitas phenylpyruvica." Hoppe-Seylers Z. Physiol. Chem. 254: 115 - 116.

[3] Folling, A. and Sydnes, S. (1958). "A method for the estimation of phenylpyruvic acid in urine with some examples of its use in dietary treatment of phenylpyruvic oligophrenia." Scandinavian journal of clinical and laboratory investigation 10(4): 355-358.

[4] Penrose, L. S. (1935). "Inheritance of phenylpyruvic amentia (phenylketonuria)." Lancet 2: 192 - 194.

[5] Scriver, C. R. (2007). "The PAH gene, phenylketonuria, and a paradigm shift." Hum Mutat 28(9): 831-845.

[6] Bickel, H. (1954). "The effects of a phenylalanine-free and phenylalanine-poor diet in phenylpyruvic oligophrenis." Experimental medicine and surgery 12(1): 114-117.

[7] Bickel, H., Gerrard, J., et al. (1954). "The influence of phenylalanine intake on the chemistry and behaviour of a phenyl-ketonuric child." Acta paediatrica 43(1): 64-77.

[8] Woolf, L. I. and Vulliamy, D. G. (1951). "Phenylketonuria with a study of the effect upon it of glutamic acid." Archives of disease in childhood 26(130): 487-494.

[9] Closs, K. and Dick Henriksen, S. (1938). "Über den Nachweis von d- und l-Phenylalanin in biologischen Flüssigkeiten." Hoppe-Seylers Z. Physiol. Chem. 254: 107 - 114.

[10] Guthrie, R. and Susi, A. (1963). "A Simple Phenylalanine Method for Detecting Phenylketonuria in Large Populations of Newborn Infants." Pediatrics 32: 338-343.

[11] Jervis, G. A. (1953). "Phenylpyruvic oligophrenia deficiency of phenylalanine-oxidizing system." Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine 82(3): 514-515.

[12] Woo, S. L., Lidsky, A. S., et al. (1983). "Cloned human phenylalanine hydroxylase gene allows prenatal diagnosis and carrier detection of classical phenylketonuria." Nature 306(5939): 151-155.

[13] Kaufman, S. (1963). "The Structure of the Phenylalanine-Hydroxylation Cofactor." Proceedings of the National Academy of Sciences of the United States of America 50: 1085-1093.

[14] Milstien, S. and Kaufman, S. (1975). "Studies on the phenylalanine hydroxylase system in liver slices." The Journal of biological chemistry 250(12): 4777-4781.

[15] Niederwieser, A. and Curtius, H. C. (1985). Tetrahydrobiopterin deficiencies in hyperphenylalaninemia. Inherited diseases of amino acid metabolism. H. Bickel and U. Wachtel. Stuttgart, Georg Thieme**:** 104 - 121.

[16] Blau, N., van Spronsen, F. J., et al. (2010). "Phenylketonuria." Lancet 376(9750): 1417-1427.

[17] Zschocke, J., Mallory, J. P., et al. (1997). "Phenylketonuria and the peoples of Northern Ireland." Human genetics 100(2): 189-194.

[18] Guldberg, P., Henriksen, K. F., et al. (1995). "Phenylketonuria in a low incidence population: molecular characterisation of mutations in Finland." Journal of medical genetics 32(12): 976-978.

[19] (2001). "National Institutes of Health Consensus Development Conference Statement: phenylketonuria: screening and management, October 16-18, 2000." Pediatrics 108(4): 972-982.

[20] Borrajo, G. J. (2007). "Newborn screening in Latin America at the beginning of the 21st century." Journal of inherited metabolic disease 30(4): 466-481.

[21] (2000). "Phenylketonuria (PKU): screening and management." NIH consensus statement 17(3): 1-33.

[22] Desviat, L. R., Perez, B., et al. (1999). "Genetic and phenotypic aspects of phenylalanine hydroxylase deficiency in Spain: molecular survey by regions." European journal of human genetics : EJHG 7(3): 386-392.

[23] Jiang, J., Ma, X., et al. (2003). "A survey for the incidence of phenylketonuria in Guangdong, China." The Southeast Asian journal of tropical medicine and public health 34 Suppl 3: 185.

[24] Zhan, J. Y., Qin, Y. F., et al. (2009). "Neonatal screening for congenital hypothyroidism and phenylketonuria in China." World journal of pediatrics : WJP 5(2): 136-139.

[25] Aoki, K., Ohwada, M., et al. (2007). "Long-term follow-up study of patients with phenylketonuria detected by the newborn screening programme in Japan." Journal of inherited metabolic disease 30(4): 608.

[26] Pangkanon, S., Charoensiriwatana, W., et al. (2009). "Detection of phenylketonuria by the newborn screening program in Thailand." The Southeast Asian journal of tropical medicine and public health 40(3): 525-529.

[27] Kang, E. S., Kaufman, S., et al. (1970). "Clinical and biochemical observations of patients with atypical phenylketonuria." Pediatrics 45(1): 83-92.

[28] Berry, H. K., Hsieh, M. H., et al. (1982). "Diagnosis of phenylalanine hydroxylase deficiency (phenylketonuria)." American journal of diseases of children 136(2): 111-114.

[29] Bartholome, K., Lutz, P., et al. (1975). "Determination of phenylalanine hydroxylase activity in patients with phenylketonuria and hyperphenylalaninemia." Pediatr Res 9(12): 899-903.

[30] Chace, D. H., Millington, D. S., et al. (1993). "Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry." Clinical chemistry 39(1): 66-71.

[31] Lukacs, Z. and Santer, R. (2006). "Evaluation of electrospray-tandem mass spectrometry for the detection of phenylketonuria and other rare disorders." Molecular nutrition & food research 50(4-5): 443-450.

[32] Fingerhut, R., Stehn, M., et al. (1997). "Comparison of four different phenylalanine determination methods." Clinica chimica acta; international journal of clinical chemistry 264(1): 65-73.

[33] Dhondt, J.-L. (2006). Laboratory diagnosis of phenylketonuria. PKU and BH4: Advances in Phenylketonuria and Tetrahydrobiopterin. N. Blau. Heilbronn, SPS Verlagsgesellschaft**:** 161-179.

[34] Marsden, D. and Levy, H. (2006). Classification of PKU. PKU and BH4: Advances in Phenylketonuria and Tetrahydrobiopterin. N. Blau. Heilbronn, SPS Verlagsgesellschaft**:** 92-103.

[35] Hsia, D. Y. (1970). "Phenylketonuria and its variants." Progress in medical genetics 7: 29-68.

[36] Kennedy, J. L., Jr., Wertelecki, W., et al. (1967). "The early treatment of phenylketonuria." American journal of diseases of children 113(1): 16-21.

[37] Blaskovics, M. E., Schaeffler, G. E., et al. (1974). "Phenylalaninaemia. Differential diagnosis." Archives of disease in childhood 49(11): 835-843.

[38] Cunningham, G. C., Day, R. W., et al. (1969). "Phenylalanine tolerance tests. In families with phenylketonuria and hyperphenylalaninemia." American journal of diseases of children 117(6): 626-635.

[39] Guttler, F. and Hansen, G. (1977). "Different phenotypes for phenylalanine hydroxylase deficiency." Annals of clinical biochemistry 14(3): 124-134.

[40] Benit, P., Rey, F., et al. (1999). "The mutant genotype is the main determinant of the metabolic phenotype in phenylalanine hydroxylase deficiency." Molecular Genetics and Metabolism 68(1): 43-47.

[41] Kayaalp, E., Treacy, E., et al. (1997). "Human phenylalanine hydroxylase mutations and hyperphenylalaninemia phenotypes: a metanalysis of genotype-phenotype correlations." American journal of human genetics 61(6): 1309-1317.

[42] Guldberg, P., Rey, F., et al. (1998). "A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype." American journal of human genetics 63(1): 71-79.

[43] Majewski, J., Schwartzentruber, J., et al. (2011). "What can exome sequencing do for you?" Journal of medical genetics 48(9): 580-589.

[44] Kuhlenbaumer, G., Hullmann, J., et al. (2011). "Novel genomic techniques open new avenues in the analysis of monogenic disorders." Human mutation 32(2): 144-151.

[45] Ng, S. B., Nickerson, D. A., et al. (2010). "Massively parallel sequencing and rare disease." Human molecular genetics 19(R2): R119-124.

[46] Gilbert, S. J. and Burgess, P. W. (2008). "Executive function." Current biology : CB 18(3): R110-114.

[47] Janzen, D. and Nguyen, M. (2010). "Beyond executive function: non-executive cognitive abilities in individuals with PKU." Molecular Genetics and Metabolism 99 Suppl 1: S47-51.

[48] Gentile, J. K., Ten Hoedt, A. E., et al. (2010). "Psychosocial aspects of PKU: hidden disabilities--a review." Molecular Genetics and Metabolism 99 Suppl 1: S64-67.

[49] Albrecht, J., Garbade, S. F., et al. (2009). "Neuropsychological speed tests and blood phenylalanine levels in patients with phenylketonuria: a meta-analysis." Neuroscience and biobehavioral reviews 33(3): 414-421.

[50] van Spronsen, F. J. and Burgard, P. (2008). "The truth of treating patients with phenylketonuria after childhood: the need for a new guideline." Journal of inherited metabolic disease 31(6): 673-679.

[51] Smith, I. and Knowles, J. (2000). "Behaviour in early treated phenylketonuria: a systematic review." European journal of pediatrics 159 Suppl 2: S89-93.

[52] Pietz, J., Fatkenheuer, B., et al. (1997). "Psychiatric disorders in adult patients with early-treated phenylketonuria." Pediatrics 99(3): 345-350.

[53] Weglage, J., Funders, B., et al. (1992). "Psychological and social findings in adolescents with phenylketonuria." European journal of pediatrics 151(7): 522-525.

[54] Waisbren, S. E. and Zaff, J. (1994). "Personality disorder in young women with treated phenylketonuria." J Inherit Metab Dis 17(5): 584-592.

[55] Mazur, A., Jarochowicz, S., et al. (2010). "Evaluation of somatic development in adult patients with previously undiagnosed and/or untreated phenylketonuria."

Medical principles and practice : international journal of the Kuwait University, Health Science Centre 19(1): 46-50.

[56] Fitzpatrick, T. B. and Miyamoto, M. (1957). "Competitive inhibition of mammalian tyrosinase by phenylalanine and its relationship to hair pigmentation in phenylketonuria." Nature 179(4552): 199-200.

[57] Yannicelli, S. and Ryan, A. (1995). "Improvements in behaviour and physical manifestations in previously untreated adults with phenylketonuria using a phenylalanine-restricted diet: a national survey." J Inherit Metab Dis 18(2): 131-134.

[58] Ramus, S. J., Forrest, S. M., et al. (1993). "Comparison of genotype and intellectual phenotype in untreated PKU patients." Journal of medical genetics 30(5): 401-405.

[59] Primrose, D. A. (1983). "Phenylketonuria with normal intelligence." Journal of mental deficiency research 27 (Pt 4): 239-246.

[60] Weglage, J., Wiedermann, D., et al. (1998). "Pathogenesis of different clinical outcomes in spite of identical genotypes and comparable blood phenylalanine concentrations in phenylketonurics." J Inherit Metab Dis 21(2): 181-182.

[61] Weglage, J., Moller, H. E., et al. (1998). "In vivo NMR spectroscopy in patients with phenylketonuria: clinical significance of interindividual differences in brain phenylalanine concentrations." J Inherit Metab Dis 21(1): 81-82.

[62] Moller, H. E., Weglage, J., et al. (1998). "Blood-brain barrier phenylalanine transport and individual vulnerability in phenylketonuria." Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 18(11): 1184-1191.

[63] Weglage, J., Pietsch, M., et al. (2001). "Normal clinical outcome in untreated subjects with mild hyperphenylalaninemia." Pediatric research 49(4): 532-536.

[64] Surtees, R. and Blau, N. (2000). "The neurochemistry of phenylketonuria." European journal of pediatrics 159 Suppl 2: S109-113.

[65] van Spronsen, F. J., Hoeksma, M., et al. (2009). "Brain dysfunction in phenylketonuria: is phenylalanine toxicity the only possible cause?" J Inherit Metab Dis 32(1): 46-51.

[66] de Groot, M. J., Hoeksma, M., et al. (2010). "Pathogenesis of cognitive dysfunction in phenylketonuria: review of hypotheses." Molecular Genetics and Metabolism 99 Suppl 1: S86-89.

[67] Smith, Q. R., Momma, S., et al. (1987). "Kinetics of neutral amino acid transport across the blood-brain barrier." Journal of neurochemistry 49(5): 1651-1658.

[68] Pardridge, W. M. (1998). "Blood-brain barrier carrier-mediated transport and brain metabolism of amino acids." Neurochemical research 23(5): 635-644.

[69] Boado, R. J., Li, J. Y., et al. (1999). "Selective expression of the large neutral amino acid transporter at the blood-brain barrier." Proceedings of the National Academy of Sciences of the United States of America 96(21): 12079-12084.

[70] Zielke, H. R., Zielke, C. L., et al. (2002). "Large neutral amino acids auto exchange when infused by microdialysis into the rat brain: implication for maple syrup urine disease and phenylketonuria." Neurochemistry international 40(4): 347-354.

[71] Pietz, J., Kreis, R., et al. (1999). "Large neutral amino acids block phenylalanine transport into brain tissue in patients with phenylketonuria." The Journal of clinical investigation 103(8): 1169-1178.

[72] Hoeksma, M., Reijngoud, D. J., et al. (2009). "Phenylketonuria: High plasma phenylalanine decreases cerebral protein synthesis." Molecular Genetics and Metabolism 96(4): 177-182.

[73] Nishihira, T., Takagi, T., et al. (1993). "Amino acid imbalance and intracellular protein synthesis." Nutrition 9(1): 37-42.

[74] Paans, A. M., Pruim, J., et al. (1996). "Neurotransmitter positron emission tomographic-studies in adults with phenylketonuria, a pilot study." European journal of pediatrics 155 Suppl 1: S78-81.

[75] Ramakers, G. J. (2002). "Rho proteins, mental retardation and the cellular basis of cognition." Trends in neurosciences 25(4): 191-199.

[76] Johnston, M. V. (2003). "Brain plasticity in paediatric neurology." European journal of paediatric neurology : EJPN : official journal of the European Paediatric Neurology Society 7(3): 105-113.

[77] Bauman, M. L. and Kemper, T. L. (1982). "Morphologic and histoanatomic observations of the brain in untreated human phenylketonuria." Acta neuropathologica 58(1): 55-63.

[78] Anderson, P. J. and Leuzzi, V. (2010). "White matter pathology in phenylketonuria." Molecular genetics and metabolism 99 Suppl 1: S3-9.

[79] Dhondt, J. L., Dautrevaux, M., et al. (1977). "A new experimental model of hyperphenylalaninemia in rat. Effect of p-chlorophenylalanine and cotrimoxazole." Biochimie 59(8-9): 713-717.

[80] Hommes, F. A., Eller, A. G., et al. (1982). "Turnover of the fast components of myelin and myelin proteins in experimental hyperphenylalaninaemia. Relevance to termination of dietary treatment in human phenylketonuria." Journal of inherited metabolic disease 5(1): 21-27.

[81] Dyer, C. A., Kendler, A., et al. (1996). "Evidence for central nervous system glial cell plasticity in phenylketonuria." Journal of neuropathology and experimental neurology 55(7): 795-814.

[82] Berger, R., Springer, J., et al. (1980). "Brain protein and myelin metabolism in hyperphenylalaninemic rats." Cellular and molecular biology, including cyto-enzymology 26(1): 31-36.

[83] Jones, S. J., Turano, G., et al. (1995). "Visual evoked potentials in phenylketonuria: association with brain MRI, dietary state, and IQ." Journal of neurology, neurosurgery, and psychiatry 59(3): 260-265.

[84] Ribas, G. S., Sitta, A., et al. (2011). "Oxidative stress in phenylketonuria: what is the evidence?" Cellular and molecular neurobiology 31(5): 653-662.

[85] Rocha, J. C. and Martins, M. J. (2012). "Oxidative stress in Phenylketonuria: future directions." J Inherit Metab Dis 35(3): 381-398.

[86] Sitta, A., Barschak, A. G., et al. (2009). "Effect of short- and long-term exposition to high phenylalanine blood levels on oxidative damage in phenylketonuric patients." International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience 27(3): 243-247.

[87] Ercal, N., Aykin-Burns, N., et al. (2002). "Oxidative stress in a phenylketonuria animal model." Free radical biology & medicine 32(9): 906-911.

[88] Sirtori, L. R., Dutra-Filho, C. S., et al. (2005). "Oxidative stress in patients with phenylketonuria." Biochim Biophys Acta 1740(1): 68-73.

[89] Ghozlan, A., Varoquaux, O., et al. (2004). "Is monoamine oxydase-B a modifying gene and phenylethylamine a harmful compound in phenylketonuria?" Molecular genetics and metabolism 83(4): 337-340.

[90] Shefer, S., Tint, G. S., et al. (2000). "Is there a relationship between 3-hydroxy-3-methylglutaryl coenzyme a reductase activity and forebrain pathology in the PKU mouse?" Journal of neuroscience research 61(5): 549-563.

[91] Martynyuk, A. E., Glushakov, A. V., et al. (2005). "Impaired glutamatergic synaptic transmission in the PKU brain." Molecular genetics and metabolism 86 Suppl 1: S34-42.

[92] Glushakov, A. V., Glushakova, O., et al. (2005). "Long-term changes in glutamatergic synaptic transmission in phenylketonuria." Brain : a journal of neurology 128(Pt 2): 300-307.

[93] Horster, F., Schwab, M. A., et al. (2006). "Phenylalanine reduces synaptic density in mixed cortical cultures from mice." Pediatric research 59(4 Pt 1): 544-548.

[94] Feillet, F., van Spronsen, F. J., et al. (2010). "Challenges and pitfalls in the management of phenylketonuria." Pediatrics 126(2): 333-341.

[95] van Spronsen, F. J., Smit, P. G., et al. (2001). "Phenylketonuria: tyrosine beyond the phenylalanine-restricted diet." J Inherit Metab Dis 24(1): 1-4.

[96] Smith, M. L., Hanley, W. B., et al. (1998). "Randomised controlled trial of tyrosine supplementation on neuropsychological performance in phenylketonuria." Archives of disease in childhood 78(2): 116-121.

[97] Butler, I. J., O'Flynn, M. E., et al. (1981). "Neurotransmitter defects and treatment of disorders of hyperphenylalaninemia." The Journal of pediatrics 98(5): 729-733.

[98] Curtius, H. C., Baerlocher, K., et al. (1972). "Pathogenesis of phenylketonuria: inhibition of DOPA and catecholamine synthesis in patients with phenylketonuria." Clinica chimica acta; international journal of clinical chemistry 42(1): 235-239.

[99] Lykkelund, C., Nielsen, J. B., et al. (1988). "Increased neurotransmitter biosynthesis in phenylketonuria induced by phenylalanine restriction or by supplementation of unrestricted diet with large amounts of tyrosine." European journal of pediatrics 148(3): 238-245.

[100] McKean, C. M. (1972). "The effects of high phenylalanine concentrations on serotonin and catecholamine metabolism in the human brain." Brain research 47(2): 469-476.

[101] Lou, H. (1985). "Large doses of tryptophan and tyrosine as potential therapeutic alternative to dietary phenylalanine restriction in phenylketonuria." Lancet 2(8447): 150-151.

[102] Joseph, B. and Dyer, C. A. (2003). "Relationship between myelin production and dopamine synthesis in the PKU mouse brain." Journal of neurochemistry 86(3): 615-626.

[103] Pascucci, T., Ventura, R., et al. (2002). "Deficits in brain serotonin synthesis in a genetic mouse model of phenylketonuria." Neuroreport 13(18): 2561-2564.

[104] Ogawa, S. and Ichinose, H. (2006). "Effect of metals and phenylalanine on the activity of human tryptophan hydroxylase-2: comparison with that on tyrosine hydroxylase activity." Neuroscience letters 401(3): 261-265.

[105] Fernstrom, J. D. and Fernstrom, M. H. (2007). "Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain." The Journal of nutrition 137(6 Suppl 1): 1539S-1547S; discussion 1548S.

[106] Burlina, A. B., Bonafe, L., et al. (2000). "Measurement of neurotransmitter metabolites in the cerebrospinal fluid of phenylketonuric patients under dietary treatment." Journal of inherited metabolic disease 23(4): 313-316.

[107] Enns, G. M., Koch, R., et al. (2010). "Suboptimal outcomes in patients with PKU treated early with diet alone: revisiting the evidence." Molecular genetics and metabolism 101(2-3): 99-109.

[108] Olsson, G. M., Montgomery, S. M., et al. (2007). "Family conditions and dietary control in phenylketonuria." Journal of inherited metabolic disease 30(5): 708-715.

[109] MacDonald, A., Gokmen-Ozel, H., et al. (2010). "The reality of dietary compliance in the management of phenylketonuria." Journal of inherited metabolic disease 33(6): 665-670.

[110] Schulz, B. and Bremer, H. J. (1995). "Nutrient intake and food consumption of adolescents and young adults with phenylketonuria." Acta paediatrica 84(7): 743-748.

[111] Bilginsoy, C., Waitzman, N., et al. (2005). "Living with phenylketonuria: perspectives of patients and their families." Journal of inherited metabolic disease 28(5): 639-649.

[112] Levy, H. L., Shih, V. E., et al. (1971). "Persistent mild hyperphenylalaninemia in the untreated state. A prospective study." The New England journal of medicine 285(8): 424-429.

[113] Belanger-Quintana, A., Burlina, A., et al. (2011). "Up to date knowledge on different treatment strategies for phenylketonuria." Molecular genetics and metabolism 104 Suppl: S19-25.

[114] McDonald, J. D. (1994). "The PKU mouse project: its history, potential and implications." Acta paediatrica 407: 122-123.

[115] Russell, W. L., Kelly, E. M., et al. (1979). "Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse." Proceedings of the National Academy of Sciences of the United States of America 76(11): 5818-5819.

[116] McDonald, J. D., Bode, V. C., et al. (1990). "Pahhph-5: a mouse mutant deficient in phenylalanine hydroxylase." Proceedings of the National Academy of Sciences of the United States of America 87(5): 1965-1967.

[117] Shedlovsky, A., Mcdonald, J. D., et al. (1993). "Mouse Models of Human Phenylketonuria." Genetics 134(4): 1205-1210.

[118] McDonald, J. D. and Charlton, C. K. (1997). "Characterization of mutations at the mouse phenylalanine hydroxylase locus." Genomics 39(3): 402-405.

[119] Takarada, Y., Kalanin, J., et al. (1993). "Phenylketonuria mutant alleles in different populations: missense mutation in exon 7 of phenylalanine hydroxylase gene." Clinical chemistry 39(11 Pt 1): 2354-2355.

[120] Sarkissian, C. N., Boulais, D. M., et al. (2000). "A heteroallelic mutant mouse model: A new orthologue for human hyperphenylalaninemia." Molecular genetics and metabolism 69(3): 188-194.

[121] Gersting, S. W., Lagler, F. B., et al. (2010). "Pahenu1 is a mouse model for tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency and promotes analysis of the pharmacological chaperone mechanism in vivo." Human molecular genetics 19(10): 2039-2049.

[122] Lagler, F. B., Gersting, S. W., et al. (2010). "New insights into tetrahydrobiopterin pharmacodynamics from Pah enu1/2, a mouse model for compound heterozygous tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency." Biochemical pharmacology 80(10): 1563-1571.

[123] Blau, N., Belanger-Quintana, A., et al. (2010). "Management of phenylketonuria in Europe: survey results from 19 countries." Molecular Genetics and Metabolism 99(2): 109-115.

[124] Fisch, R. O., Matalon, R., et al. (1997). "Phenylketonuria: current dietary treatment practices in the United States and Canada." Journal of the American College of Nutrition 16(2): 147-151.

[125] Gassio, R., Campistol, J., et al. (2003). "Do adult patients with phenylketonuria improve their quality of life after introduction/resumption of a phenylalanine-restricted diet?" Acta paediatrica 92(12): 1474-1478.

[126] Andersen, A. E. and Avins, L. (1976). "Lowering brain phenylalanine levels by giving other large neutral amino acids. A new experimental therapeutic approach to phenylketonuria." Archives of neurology 33(10): 684-686.

[127] Koch, R., Moseley, K. D., et al. (2003). "Large neutral amino acid therapy and phenylketonuria: a promising approach to treatment." Molecular genetics and metabolism 79(2): 110-113.

[128] Moats, R. A., Moseley, K. D., et al. (2003). "Brain phenylalanine concentrations in phenylketonuria: research and treatment of adults." Pediatrics 112(6 Pt 2): 1575-1579.

[129] Matalon, R., Michals-Matalon, K., et al. (2007). "Double blind placebo control trial of large neutral amino acids in treatment of PKU: effect on blood phenylalanine." Journal of inherited metabolic disease 30(2): 153-158.

[130] Schindeler, S., Ghosh-Jerath, S., et al. (2007). "The effects of large neutral amino acid supplements in PKU: an MRS and neuropsychological study." Molecular genetics and metabolism 91(1): 48-54.

[131] Laclair, C. E., Ney, D. M., et al. (2009). "Purification and use of glycomacropeptide for nutritional management of phenylketonuria." Journal of food science 74(4): E199-206.

[132] Lim, K., van Calcar, S. C., et al. (2007). "Acceptable low-phenylalanine foods and beverages can be made with glycomacropeptide from cheese whey for individuals with PKU." Molecular genetics and metabolism 92(1-2): 176-178.

[133] Ney, D. M., Gleason, S. T., et al. (2009). "Nutritional management of PKU with glycomacropeptide from cheese whey." Journal of inherited metabolic disease 32(1): 32-39.

[134] van Calcar, S. C., MacLeod, E. L., et al. (2009). "Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids." The American journal of clinical nutrition 89(4): 1068-1077.

[135] Hodgins, D. S. (1971). "Yeast phenylalanine ammonia-lyase. Purification, properties, and the identification of catalytically essential dehydroalanine." The Journal of biological chemistry 246(9): 2977-2985.

[136] Sarkissian, C. N., Shao, Z., et al. (1999). "A different approach to treatment of phenylketonuria: phenylalanine degradation with recombinant phenylalanine ammonia lyase." Proceedings of the National Academy of Sciences of the United States of America 96(5): 2339-2344.

[137] Hoskins, J. A., Holliday, S. B., et al. (1984). "The metabolism of cinnamic acid by healthy and phenylketonuric adults: a kinetic study." Biomedical mass spectrometry 11(6): 296-300.

[138] Kim, W., Erlandsen, H., et al. (2004). "Trends in enzyme therapy for phenylketonuria." Molecular therapy : the journal of the American Society of Gene Therapy 10(2): 220-224.

[139] Sarkissian, C. N., Gamez, A., et al. (2008). "Preclinical evaluation of multiple species of PEGylated recombinant phenylalanine ammonia lyase for the treatment of

phenylketonuria." Proceedings of the National Academy of Sciences of the United States of America 105(52): 20894-20899.

[140] Wang, L., Gamez, A., et al. (2008). "Structural and biochemical characterization of the therapeutic Anabaena variabilis phenylalanine ammonia lyase." Journal of molecular biology 380(4): 623-635.

[141] Gamez, A., Wang, L., et al. (2007). "Structure-based epitope and PEGylation sites mapping of phenylalanine ammonia-lyase for enzyme substitution treatment of phenylketonuria." Molecular genetics and metabolism 91(4): 325-334.

[142] Kang, T. S., Wang, L., et al. (2010). "Converting an injectable protein therapeutic into an oral form: phenylalanine ammonia lyase for phenylketonuria." Molecular genetics and metabolism 99(1): 4-9.

[143] Ding, Z., Georgiev, P., et al. (2006). "Administration-route and gender-independent long-term therapeutic correction of phenylketonuria (PKU) in a mouse model by recombinant adeno-associated virus 8 pseudotyped vector-mediated gene transfer." Gene Therapy 13(7): 587-593.

[144] Harding, C. O., Gillingham, M. B., et al. (2006). "Complete correction of hyperphenylalaninemia following liver-directed, recombinant AAV2/8 vector-mediated gene therapy in murine phenylketonuria." Gene Therapy 13(5): 457-462.

[145] Ding, Z., Harding, C. O., et al. (2008). "Correction of murine PKU following AAV-mediated intramuscular expression of a complete phenylalanine hydroxylating system." Molecular therapy : the journal of the American Society of Gene Therapy 16(4): 673-681.

[146] Thony, B. (2010). "Long-term correction of murine phenylketonuria by viral gene transfer: liver versus muscle." Journal of inherited metabolic disease 33(6): 677-680.

[147] Viecelli, H. M., Harbottle, R. P., et al. (2012). "Treatment of phenylketonuria (PKU) using minicircle-based naked-DNA gene transfer to murine liver." Manuscript in preparation.

[148] Harding, C. (2008). "Progress toward cell-directed therapy for phenylketonuria." Clinical genetics 74(2): 97-104.

[149] Niederwieser, A., Curtius, H. C., et al. (1982). "Atypical phenylketonuria with defective biopterin metabolism. Monotherapy with tetrahydrobiopterin or sepiapterin, screening and study of biosynthesis in man." European journal of pediatrics 138(2): 110-112.

[150] Kure, S., Hou, D. C., et al. (1999). "Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency." J Pediatr 135(3): 375-378.

[151] Fiege, B. and Blau, N. (2007). "Assessment of tetrahydrobiopterin (BH4) responsiveness in phenylketonuria." The Journal of pediatrics 150(6): 627-630.

[152] Trefz, F. K., Aulela-Scholz, C., et al. (2001). "Successful treatment of phenylketonuria with tetrahydrobiopterin." European journal of pediatrics 160(5): 315.

[153] Bernegger, C. and Blau, N. (2002). "High frequency of tetrahydrobiopterin-responsiveness among hyperphenylalaninemias: a study of 1,919 patients observed from 1988 to 2002." Molecular genetics and metabolism 77(4): 304-313.

[154] Blau, N. (2010). "Phenylketonuria and BH4 Deficiencies." Uni-Med Science.

[155] Trefz, F. K., Burton, B. K., et al. (2009). "Efficacy of sapropterin dihydrochloride in increasing phenylalanine tolerance in children with phenylketonuria: a phase III, randomized, double-blind, placebo-controlled study." J Pediatr 154(5): 700-707.

[156] Burton, B. K., Grange, D. K., et al. (2007). "The response of patients with phenylketonuria and elevated serum phenylalanine to treatment with oral sapropterin dihydrochloride (6R-tetrahydrobiopterin): a phase II, multicentre, open-label, screening study." Journal of inherited metabolic disease 30(5): 700-707.

[157] Levy, H. L., Milanowski, A., et al. (2007). "Efficacy of sapropterin dihydrochloride (tetrahydrobiopterin, 6R-BH4) for reduction of phenylalanine concentration in patients with phenylketonuria: a phase III randomised placebo-controlled study." Lancet 370(9586): 504-510.

[158] Lambruschini, N., Perez-Duenas, B., et al. (2005). "Clinical and nutritional evaluation of phenylketonuric patients on tetrahydrobiopterin monotherapy." Molecular genetics and metabolism 86 Suppl 1: S54-60.

[159] Zurfluh, M. R., Zschocke, J., et al. (2008). "Molecular genetics of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency." Human mutation 29(1): 167-175.

[160] Muntau, A. C., Roschinger, W., et al. (2002). "Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria." N Engl J Med 347(26): 2122-2132.

[161] Cohen, F. E. and Kelly, J. W. (2003). "Therapeutic approaches to protein-misfolding diseases." Nature 426(6968): 905-909.

[162] Loo, T. W. and Clarke, D. M. (2007). "Chemical and pharmacological chaperones as new therapeutic agents." Expert reviews in molecular medicine 9(16): 1-18.

[163] Pey, A. L., Ying, M., et al. (2008). "Identification of pharmacological chaperones as potential therapeutic agents to treat phenylketonuria." J Clin Invest 118(8): 2858-2867.

[164] Santos-Sierra, S., Kirchmair, J., et al. (2012). "Novel pharmacological chaperones that correct phenylketonuria in mice." Human molecular genetics.

[165] van Spronsen, F. J. (2010). "Phenylketonuria: a 21st century perspective." Nature reviews. Endocrinology 6(9): 509-514.

[166] Kaufman, S. (1993). "The phenylalanine hydroxylating system." Advances in enzymology and related areas of molecular biology 67: 77-264.

[167] Lichter-Konecki, U., Hipke, C. M., et al. (1999). "Human phenylalanine hydroxylase gene expression in kidney and other nonhepatic tissues." Molecular genetics and metabolism 67(4): 308-316.

[168] Moller, N., Meek, S., et al. (2000). "The kidney is an important site for in vivo phenylalanine-to-tyrosine conversion in adult humans: A metabolic role of the kidney." Proceedings of the National Academy of Sciences of the United States of America 97(3): 1242-1246.

[169] Tessari, P., Deferrari, G., et al. (1999). "Phenylalanine hydroxylation across the kidney in humans rapid communication." Kidney international 56(6): 2168-2172.

[170] Fitzpatrick, P. F. (2011). "Allosteric regulation of phenylalanine hydroxylase." Archives of biochemistry and biophysics.

[171] Hufton, S. E., Jennings, I. G., et al. (1995). "Structure and function of the aromatic amino acid hydroxylases." The Biochemical journal 311 (Pt 2): 353-366.

[172] Kappock, T. J. and Caradonna, J. P. (1996). "Pterin-Dependent Amino Acid Hydroxylases." Chemical Reviews 96(7): 2659 - 2756.

[173] Fitzpatrick, P. F. (2000). "The aromatic amino acid hydroxylases." Advances in enzymology and related areas of molecular biology 74: 235-294.

[174] Pey, A. L. and Martinez, A. (2006). The Phenylalanine Hydroxylase System. PKU and BH4: Advances in Phenylketonuria and Tetrahydrobiopterin. N. Blau. Heilbronn, SPS Verlagsgesellschaft**:** 67-91.

[175] Scriver, C. R., Levy, H., et al. (2001). Hyperphenylalaninemia: Phenylalanine Hydroxylase Deficiency. The Molecular and Metabolic Bases of Inherited Disease. S. C.R., D. Valle, A. L. Beaudetet al. New York, Mc Graw-Hill Professional.

[176] Konecki, D. S., Wang, Y., et al. (1992). "Structural characterization of the 5' regions of the human phenylalanine hydroxylase gene." Biochemistry 31(35): 8363-8368.

[177] Kwok, S. C., Ledley, F. D., et al. (1985). "Nucleotide sequence of a full-length complementary DNA clone and amino acid sequence of human phenylalanine hydroxylase." Biochemistry 24(3): 556-561.

[178] Scriver, C. R., Byck, S., et al. (1996). "The phenylalanine hydroxylase locus: a marker for the history of phenylketonuria and human genetic diversity. PAH Mutation Analysis Consortium." Ciba Foundation symposium 197: 73-90; discussion 90-76.

[179] Hoang, L., Byck, S., et al. (1996). "PAH Mutation Analysis Consortium Database: a database for disease-producing and other allelic variation at the human PAH locus." Nucleic acids research 24(1): 127-131.

[180] Scriver, C. R., Hurtubise, M., et al. (2003). "PAHdb 2003: what a locus-specific knowledgebase can do." Hum Mutat 21(4): 333-344.

[181] Ledley, F. D., Grenett, H. E., et al. (1990). "Mouse phenylalanine hydroxylase. Homology and divergence from human phenylalanine hydroxylase." The Biochemical journal 267(2): 399-405.

[182] Davis, M. D., Parniak, M. A., et al. (1997). "The role of phenylalanine in structure-function relationships of phenylalanine hydroxylase revealed by radiation target analysis." Proceedings of the National Academy of Sciences of the United States of America 94(2): 491-495.

[183] Martinez, A., Knappskog, P. M., et al. (1995). "Expression of recombinant human phenylalanine hydroxylase as fusion protein in Escherichia coli circumvents proteolytic degradation by host cell proteases. Isolation and characterization of the wild-type enzyme." Biochem J 306 (Pt 2): 589-597.

[184] Celikel, R., Davis, M. D., et al. (1991). "Crystallization and preliminary X-ray analysis of phenylalanine hydroxylase from rat liver." Journal of molecular biology 218(3): 495-498.

[185] Kaufman, S. and Fisher, D. B. (1970). "Purification and some physical properties of phenylalanine hydroxylase from rat liver." The Journal of biological chemistry 245(18): 4745-4750.

[186] Tourian, A. (1971). "Activation of phenylalanine hydroxylase by phenylalanine." Biochim Biophys Acta 242(2): 345-354.

[187] Kappock, T. J., Harkins, P. C., et al. (1995). "Spectroscopic and kinetic properties of unphosphorylated rat hepatic phenylalanine hydroxylase expressed in Escherichia coli. Comparison of resting and activated states." The Journal of biological chemistry 270(51): 30532-30544.

[188] Erlandsen, H., Fusetti, F., et al. (1997). "Crystal structure of the catalytic domain of human phenylalanine hydroxylase reveals the structural basis for phenylketonuria." Nature structural biology 4(12): 995-1000.

[189] Fusetti, F., Erlandsen, H., et al. (1998). "Structure of tetrameric human phenylalanine hydroxylase and its implications for phenylketonuria." The Journal of biological chemistry 273(27): 16962-16967.

[190] Kobe, B., Jennings, I. G., et al. (1999). "Structural basis of autoregulation of phenylalanine hydroxylase." Nature structural biology 6(5): 442-448.

[191] Goodwill, K. E., Sabatier, C., et al. (1997). "Crystal structure of tyrosine hydroxylase at 2.3 A and its implications for inherited neurodegenerative diseases." Nature structural biology 4(7): 578-585.

[192] Erlandsen, H. and Stevens, R. C. (1999). "The structural basis of phenylketonuria." Molecular Genetics and Metabolism 68(2): 103-125.

[193] Abita, J. P., Milstien, S., et al. (1976). "In vitro activation of rat liver phenylalanine hydroxylase by phosphorylation." The Journal of biological chemistry 251(17): 5310-5314.

[194] Doskeland, A. P., Schworer, C. M., et al. (1984). "Some aspects of the phosphorylation of phenylalanine 4-monooxygenase by a calcium-dependent and calmodulin-dependent protein kinase." European journal of biochemistry / FEBS 145(1): 31-37.

[195] Bailey, S. W. and Ayling, J. E. (1978). "Separation and properties of the 6-diastereoisomers of l-erythro-tetrahydrobiopterin and their reactivities with phenylalanine hydroxylase." The Journal of biological chemistry 253(5): 1598-1605.

[196] Nielsen, K. H. (1969). "Rat liver phenylalanine hydroxylase. A method for the measurement of activity, with particular reference to the distinctive features of the enzyme and the pteridine cofactor." European journal of biochemistry / FEBS 7(3): 360-369.

[197] Shiman, R. (1985). Phenylalanine hydroxylase and dihydropterin reductase. Folates and Pterins. R. L. Blakley and S. J. Benkovic. New York, Wiley and Sons. 2**:** 179-249.

[198] Mitnaul, L. J. and Shiman, R. (1995). "Coordinate regulation of tetrahydrobiopterin turnover and phenylalanine hydroxylase activity in rat liver cells." Proceedings of the National Academy of Sciences of the United States of America 92(3): 885-889.

[199] Fisher, D. B. and Kaufman, S. (1970). "The effect of enzyme concentration, ionic strength, and temperature on the stoichiometry of the phenylalanine hydroxylase reaction." Biochemical and biophysical research communications 38(4): 663-669.

[200] Doskeland, A. P., Martinez, A., et al. (1996). "Phosphorylation of recombinant human phenylalanine hydroxylase: effect on catalytic activity, substrate activation and protection against non-specific cleavage of the fusion protein by restriction protease." Biochem J 313 (Pt 2): 409-414.

[201] Thorolfsson, M., Ibarra-Molero, B., et al. (2002). "L-phenylalanine binding and domain organization in human phenylalanine hydroxylase: a differential scanning calorimetry study." Biochemistry 41(24): 7573-7585.

[202] Thorolfsson, M., Teigen, K., et al. (2003). "Activation of phenylalanine hydroxylase: effect of substitutions at Arg68 and Cys237." Biochemistry 42(12): 3419-3428.

[203] Teigen, K. and Martinez, A. (2003). "Probing cofactor specificity in phenylalanine hydroxylase by molecular dynamics simulations." Journal of biomolecular structure & dynamics 20(6): 733-740.

[204] Pey, A. L., Thorolfsson, M., et al. (2004). "Thermodynamic characterization of the binding of tetrahydropterins to phenylalanine hydroxylase." Journal of the American Chemical Society 126(42): 13670-13678.

[205] Kappock, T. J. and Caradonna, J. P. (1996). "Pterin-Dependent Amino Acid Hydroxylases." Chemical Reviews 96(7): 2659-2756.

[206] Gersting, S. W., Staudigl, M., et al. (2010). "Activation of phenylalanine hydroxylase induces positive cooperativity toward the natural cofactor." J Biol Chem 285(40): 30686-30697.

[207] Pey, A. L. and Martinez, A. (2005). "The activity of wild-type and mutant phenylalanine hydroxylase and its regulation by phenylalanine and tetrahydrobiopterin at physiological and pathological concentrations: an isothermal titration calorimetry study." Mol Genet Metab 86 Suppl 1: S43-53.

[208] Fisher, D. B., Kirkwood, R., et al. (1972). "Rat liver phenylalanine hydroxylase, an iron enzyme." The Journal of biological chemistry 247(16): 5161-5167.

[209] Gibbs, B. S., Wojchowski, D., et al. (1993). "Expression of rat liver phenylalanine hydroxylase in insect cells and site-directed mutagenesis of putative non-heme iron-binding sites." The Journal of biological chemistry 268(11): 8046-8052.

[210] Bassan, A., Blomberg, M. R., et al. (2003). "Mechanism of dioxygen cleavage in tetrahydrobiopterin-dependent amino acid hydroxylases." Chemistry 9(1): 106-115.

[211] Solomon, E. I., Decker, A., et al. (2003). "Non-heme iron enzymes: contrasts to heme catalysis." Proceedings of the National Academy of Sciences of the United States of America 100(7): 3589-3594.

[212] Fitzpatrick, P. F. (2003). "Mechanism of aromatic amino acid hydroxylation." Biochemistry 42(48): 14083-14091.

[213] Miranda, F. F., Teigen, K., et al. (2002). "Phosphorylation and mutations of Ser(16) in human phenylalanine hydroxylase. Kinetic and structural effects." The Journal of biological chemistry 277(43): 40937-40943.

[214] Stokka, A. J., Carvalho, R. N., et al. (2004). "Probing the role of crystallographically defined/predicted hinge-bending regions in the substrate-induced global conformational transition and catalytic activation of human phenylalanine hydroxylase by single-site mutagenesis." The Journal of biological chemistry 279(25): 26571-26580.

[215] Jennings, I. G., Cotton, R. G., et al. (2000). "Structural interpretation of mutations in phenylalanine hydroxylase protein aids in identifying genotype-phenotype correlations in phenylketonuria." European journal of human genetics : EJHG 8(9): 683-696.

[216] Pey, A. L., Perez, B., et al. (2004). "Mechanisms underlying responsiveness to tetrahydrobiopterin in mild phenylketonuria mutations." Hum Mutat 24(5): 388-399.

[217] Blau, N. and Erlandsen, H. (2004). "The metabolic and molecular bases of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency." Molecular Genetics and Metabolism 82(2): 101-111.

[218] Hopkins, F. G. (1942). "A contribution to the chemistry of pterins." Proc. Royal Soc. 130: 359 - 379.

[219] Blau, N., Thony, B., et al. (2001). Disorders of Tetrahydrobiopterin and Related Biogenic Amines. The Molecular and Metabolic Bases of Inherited Disease. S. C.R., D. Valle, A. L. Beaudetet al. New York, Mc Graw-Hill Professional.

[220] Niederwieser, A., Blau, N., et al. (1984). "GTP cyclohydrolase I deficiency, a new enzyme defect causing hyperphenylalaninemia with neopterin, biopterin, dopamine, and serotonin deficiencies and muscular hypotonia." European journal of pediatrics 141(4): 208-214.

[221] Harada, T., Kagamiyama, H., et al. (1993). "Feedback regulation mechanisms for the control of GTP cyclohydrolase I activity." Science 260(5113): 1507-1510.

[222] Yoneyama, T. and Hatakeyama, K. (2001). "Ligand binding to the inhibitory and stimulatory GTP cyclohydrolase I/GTP cyclohydrolase I feedback regulatory protein complexes." Protein science : a publication of the Protein Society 10(4): 871-878.

[223] Ponzone, A., Guardamagna, O., et al. (1993). "Hyperphenylalaninemia and pterin metabolism in serum and erythrocytes." Clinica chimica acta; international journal of clinical chemistry 216(1-2): 63-71.

[224] Thony, B., Auerbach, G., et al. (2000). "Tetrahydrobiopterin biosynthesis, regeneration and functions." The Biochemical journal 347 Pt 1: 1-16.

[225] Werner, E. R., Blau, N., et al. (2011). "Tetrahydrobiopterin: biochemistry and pathophysiology." The Biochemical journal 438(3): 397-414.

[226] Park, Y. S., Heizmann, C. W., et al. (1991). "Human carbonyl and aldose reductases: new catalytic functions in tetrahydrobiopterin biosynthesis." Biochemical and biophysical research communications 175(3): 738-744.

[227] Tayeh, M. A. and Marletta, M. A. (1989). "Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopterin is required as a cofactor." The Journal of biological chemistry 264(33): 19654-19658.

[228] Gorren, A. C. and Mayer, B. (2002). "Tetrahydrobiopterin in nitric oxide synthesis: a novel biological role for pteridines." Current drug metabolism 3(2): 133-157.

[229] Foxton, R. H., Land, J. M., et al. (2007). "Tetrahydrobiopterin availability in Parkinson's and Alzheimer's disease; potential pathogenic mechanisms." Neurochemical research 32(4-5): 751-756.

[230] Moens, A. L. and Kass, D. A. (2007). "Therapeutic potential of tetrahydrobiopterin for treating vascular and cardiac disease." Journal of cardiovascular pharmacology 50(3): 238-246.

[231] Tietz, A., Lindberg, M., et al. (1964). "A New Pteridine-Requiring Enzyme System for the Oxidation of Glyceryl Ethers." The Journal of biological chemistry 239: 4081-4090.

[232] Kaufman, S., Pollock, R. J., et al. (1990). "Dependence of an alkyl glycol-ether monooxygenase activity upon tetrahydropterins." Biochimica et biophysica acta 1040(1): 19-27.

[233] Watschinger, K., Keller, M. A., et al. (2010). "Identification of the gene encoding alkylglycerol monooxygenase defines a third class of tetrahydrobiopterin-dependent enzymes." Proceedings of the National Academy of Sciences of the United States of America 107(31): 13672-13677.

[234] Zurfluh, M. R., Giovannini, M., et al. (2005). "Screening for tetrahydrobiopterin deficiencies using dried blood spots on filter paper." Molecular genetics and metabolism 86 Suppl 1: S96-103.

[235] Niederwieser, A., Staudenmann, W., et al. (1984). "High-performance liquid chromatography with column switching for the analysis of biogenic amine metabolites and pterins." Journal of chromatography 290: 237-246.

[236] Opladen, T., Abu Seda, B., et al. (2011). "Diagnosis of tetrahydrobiopterin deficiency using filter paper blood spots: further development of the method and 5 years experience." Journal of inherited metabolic disease 34(3): 819-826.

[237] Blau, N., Bonafe, L., et al. (2001). "Tetrahydrobiopterin deficiencies without hyperphenylalaninemia: diagnosis and genetics of dopa-responsive dystonia and

sepiapterin reductase deficiency." Molecular genetics and metabolism 74(1-2): 172-185.

[238] Bonafe, L., Thony, B., et al. (2001). "Diagnosis of dopa-responsive dystonia and other tetrahydrobiopterin disorders by the study of biopterin metabolism in fibroblasts." Clinical chemistry 47(3): 477-485.

[239] Zorzi, G., Thony, B., et al. (2002). "Reduced nitric oxide metabolites in CSF of patients with tetrahydrobiopterin deficiency." Journal of neurochemistry 80(2): 362-364.

[240] Blau, N. (2006). Nomenclature and laboratory diagnosis of tetrahydrobiopterin deficiencies. PKU and BH4: Advances in Phenylketonuria and Tetrahydrobiopterin. N. Blau. Heilbronn, SPS Verlagsgesellschaft**:** 555 - 567.

[241] Bonafe, L., Thony, B., et al. (2001). "Mutations in the sepiapterin reductase gene cause a novel tetrahydrobiopterin-dependent monoamine-neurotransmitter deficiency without hyperphenylalaninemia." American journal of human genetics 69(2): 269-277.

[242] Friedman, J., Roze, E., et al. (2012) Sepiapterin reductase deficiency: A treatable mimic of cerebral palsy. Annals of neurology DOI: 10.1002/ana.22685

[243] Waters, P. J., Parniak, M. A., et al. (1998). "In vitro expression analysis of mutations in phenylalanine hydroxylase: linking genotype to phenotype and structure to function." Hum Mutat 11(1): 4-17.

[244] Ledley, F. D., Grenett, H. E., et al. (1987). "Biochemical characterization of recombinant human phenylalanine hydroxylase produced in Escherichia coli." J Biol Chem 262(5): 2228-2233.

[245] Eiken, H. G., Knappskog, P. M., et al. (1996). "PKU mutation G46S is associated with increased aggregation and degradation of the phenylalanine hydroxylase enzyme." Human mutation 7(3): 228-238.

[246] Bjorgo, E., Knappskog, P. M., et al. (1998). "Partial characterization and three-dimensional-structural localization of eight mutations in exon 7 of the human phenylalanine hydroxylase gene associated with phenylketonuria." Eur J Biochem 257(1): 1-10.

[247] Gamez, A., Perez, B., et al. (2000). "Expression analysis of phenylketonuria mutations. Effect on folding and stability of the phenylalanine hydroxylase protein." J Biol Chem 275(38): 29737-29742.

[248] Waters, P. J., Parniak, M. A., et al. (1999). "Missense mutations in the phenylalanine hydroxylase gene (PAH) can cause accelerated proteolytic turnover of PAH enzyme: a mechanism underlying phenylketonuria." Journal of inherited metabolic disease 22(3): 208-212.

[249] Gjetting, T., Petersen, M., et al. (2001). "In vitro expression of 34 naturally occurring mutant variants of phenylalanine hydroxylase: correlation with metabolic phenotypes and susceptibility toward protein aggregation." Molecular genetics and metabolism 72(2): 132-143.

[250] Waters, P. J., Scriver, C. R., et al. (2001). "Homomeric and heteromeric interactions between wild-type and mutant phenylalanine hydroxylase subunits: evaluation of two-hybrid approaches for functional analysis of mutations causing hyperphenylalaninemia." Molecular genetics and metabolism 73(3): 230-238.

[251] Perez, B., Desviat, L. R., et al. (2003). "Analysis of defective subunit interactions using the two-hybrid system." Methods in molecular biology 232: 245-256.

[252] Leandro, J., Nascimento, C., et al. (2006). "Co-expression of different subunits of human phenylalanine hydroxylase: evidence of negative interallelic complementation." Biochimica et biophysica acta 1762(5): 544-550.

[253] Doskeland, A. P. and Flatmark, T. (1996). "Recombinant human phenylalanine hydroxylase is a substrate for the ubiquitin-conjugating enzyme system." Biochem J 319 (Pt 3): 941-945.

[254] Bross, P., Andresen, B. S., et al. (2011). Protein Misfolding and Degradation in Genetic Disease. Encyclopedia of Life Sciences. Chichester, John Wiley & Sons.

[255] Pey, A. L., Desviat, L. R., et al. (2003). "Phenylketonuria: genotype-phenotype correlations based on expression analysis of structural and functional mutations in PAH." Hum Mutat 21(4): 370-378.

[256] Kim, S. W., Jung, J., et al. (2006). "Structural and functional analyses of mutations of the human phenylalanine hydroxylase gene." Clin Chim Acta 365(1-2): 279-287.

[257] Waters, P. J., Parniak, M. A., et al. (2000). "Characterization of phenylketonuria missense substitutions, distant from the phenylalanine hydroxylase active site, illustrates a paradigm for mechanism and potential modulation of phenotype." Mol Genet Metab 69(2): 101-110.

[258] Gregersen, N., Bross, P., et al. (2006). "Protein misfolding and human disease." Annual review of genomics and human genetics 7: 103-124.

[259] Betts, S., Haase-Pettingell, C., et al. (1997). "Mutational effects on inclusion body formation." Advances in protein chemistry 50: 243-264.

[260] Waters, P. J. (2003). "How PAH gene mutations cause hyper-phenylalaninemia and why mechanism matters: insights from in vitro expression." Hum Mutat 21(4): 357-369.

[261] Pey, A. L., Stricher, F., et al. (2007). "Predicted effects of missense mutations on native-state stability account for phenotypic outcome in phenylketonuria, a paradigm of misfolding diseases." Am J Hum Genet 81(5): 1006-1024.

[262] Gersting, S. W., Kemter, K. F., et al. (2008). "Loss of function in phenylketonuria is caused by impaired molecular motions and conformational instability." Am J Hum Genet 83(1): 5-17.

[263] Lopez-Bigas, N., Audit, B., et al. (2005). "Are splicing mutations the most frequent cause of hereditary disease?" FEBS letters 579(9): 1900-1903.

[264] Wang, G. S. and Cooper, T. A. (2007). "Splicing in disease: disruption of the splicing code and the decoding machinery." Nature reviews. Genetics 8(10): 749-761.

[265] Andresen, B. S. and Krainer, A. R. (2009). When the genetic code is not enough - How sequence variations can affect pre-mRNA splicing and cause (complex) disease. Genetics of Complex Human Diseases. L. Almasy and A. Al-Chalabi. New York, Cold Spring Harbor**:** 165-182.

[266] Ward, A. J. and Cooper, T. A. (2010). "The pathobiology of splicing." The Journal of pathology 220(2): 152-163.

[267] DiLella, A. G., Marvit, J., et al. (1986). "Tight linkage between a splicing mutation and a specific DNA haplotype in phenylketonuria." Nature 322(6082): 799-803.

[268] Marvit, J., DiLella, A. G., et al. (1987). "GT to AT transition at a splice donor site causes skipping of the preceding exon in phenylketonuria." Nucleic acids research 15(14): 5613-5628.

[269] Chelly, J., Concordet, J. P., et al. (1989). "Illegitimate transcription: transcription of any gene in any cell type." Proceedings of the National Academy of Sciences of the United States of America 86(8): 2617-2621.

[270] Sarkar, G. and Sommer, S. S. (1989). "Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity." Science 244(4902): 331-334.

[271] Abadie, V., Jaruzelska, J., et al. (1993). "Illegitimate transcription of the phenylalanine hydroxylase gene in lymphocytes for identification of mutations in phenylketonuria." Human molecular genetics 2(1): 31-34.

[272] Chan, M. A., Stein, L. D., et al. (1986). "Heterogeneity of EBV-transformable human B lymphocyte populations." Journal of immunology 136(1): 106-112.

[273] Ellingsen, S., Knappskog, P. M., et al. (1997). "Phenylketonuria splice mutation (EXON6nt-96A-->g) masquerading as missense mutation (Y204C)." Hum Mutat 9(1): 88-90.

[274] Ellingsen, S., Knappskog, P. M., et al. (1999). "Diverse PAH transcripts in lymphocytes of PKU patients with putative nonsense (G272X, Y356X) and missense (P281L, R408Q) mutations." FEBS Lett 457(3): 505-508.

[275] Cooper, T. A. (2005). "Use of minigene systems to dissect alternative splicing elements." Methods 37(4): 331-340.

[276] Chao, H. K., Hsiao, K. J., et al. (2001). "A silent mutation induces exon skipping in the phenylalanine hydroxylase gene in phenylketonuria." Hum Genet 108(1): 14-19.

[277] Dobrowolski, S. F., Andersen, H. S., et al. (2010). "The phenylalanine hydroxylase c.30C>G synonymous variation (p.G10G) creates a common exonic splicing silencer." Mol Genet Metab 100(4): 316-323.

[278] Gjetting, T., Romstad, A., et al. (2001). "A phenylalanine hydroxylase amino acid polymorphism with implications for molecular diagnostics." Molecular genetics and metabolism 73(3): 280-284.

[279] Ho, P. Y., Huang, M. Z., et al. (2008). "Simultaneous assessment of the effects of exonic mutations on RNA splicing and protein functions." Biochemical and biophysical research communications 373(4): 515-520.

[280] Bashyam, M. D., Chaudhary, A. K., et al. (2010). "Phenylalanine hydroxylase gene mutations in phenylketonuria patients from India: identification of novel mutations that affect PAH RNA." Molecular genetics and metabolism 100(1): 96-99.

[281] Cartegni, L., Chew, S. L., et al. (2002). "Listening to silence and understanding nonsense: exonic mutations that affect splicing." Nature reviews. Genetics 3(4): 285-298.

[282] Pagani, F. and Baralle, F. E. (2004). "Genomic variants in exons and introns: identifying the splicing spoilers." Nat Rev Genet 5(5): 389-396.

[283] Erlandsen, H., Patch, M. G., et al. (2003). "Structural studies on phenylalanine hydroxylase and implications toward understanding and treating phenylketonuria." Pediatrics 112(6 Pt 2): 1557-1565.

[284] Matalon, R., Koch, R., et al. (2004). "Biopterin responsive phenylalanine hydroxylase deficiency." Genetics in medicine : official journal of the American College of Medical Genetics 6(1): 27-32.

[285] Erlandsen, H., Pey, A. L., et al. (2004). "Correction of kinetic and stability defects by tetrahydrobiopterin in phenylketonuria patients with certain phenylalanine hydroxylase mutations." Proc Natl Acad Sci U S A 101(48): 16903-16908.

[286] Leandro, P., Rivera, I., et al. (2000). "The V388M mutation results in a kinetic variant form of phenylalanine hydroxylase." Molecular genetics and metabolism 69(3): 204-212.

[287] Perez, B., Desviat, L. R., et al. (2005). "Kinetic and stability analysis of PKU mutations identified in BH4-responsive patients." Molecular genetics and metabolism 86 Suppl 1: S11-16.

[288] Knappskog, P. M., Eiken, H. G., et al. (1996). "PKU mutation (D143G) associated with an apparent high residual enzyme activity: expression of a kinetic variant form of phenylalanine hydroxylase in three different systems." Hum Mutat 8(3): 236-246.

[289] Kure, S., Sato, K., et al. (2004). "Wild-type phenylalanine hydroxylase activity is enhanced by tetrahydrobiopterin supplementation in vivo: an implication for therapeutic basis of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency." Molecular genetics and metabolism 83(1-2): 150-156.

[290] Thony, B., Ding, Z., et al. (2004). "Tetrahydrobiopterin protects phenylalanine hydroxylase activity in vivo: implications for tetrahydrobiopterin-responsive hyperphenylalaninemia." FEBS letters 577(3): 507-511.

[291] Aguado, C., Perez, B., et al. (2006). "Analysis of the effect of tetrahydrobiopterin on PAH gene expression in hepatoma cells." FEBS Lett 580(7): 1697-1701.

[292] Scavelli, R., Ding, Z., et al. (2005). "Stimulation of hepatic phenylalanine hydroxylase activity but not Pah-mRNA expression upon oral loading of tetrahydrobiopterin in normal mice." Molecular genetics and metabolism 86 Suppl 1: S153-155.

[293] Staudigl, M., Gersting, S. W., et al. (2011). "The interplay between genotype, metabolic state and cofactor treatment governs phenylalanine hydroxylase function and drug response." Human molecular genetics 20(13): 2628-2641.

[294] Okano, Y., Eisensmith, R. C., et al. (1991). "Molecular basis of phenotypic heterogeneity in phenylketonuria." N Engl J Med 324(18): 1232-1238.

[295] Svensson, E., von Dobeln, U., et al. (1993). "Relation between genotype and phenotype in Swedish phenylketonuria and hyperphenylalaninemia patients." European journal of pediatrics 152(2): 132-139.

[296] Kaufman, S., Max, E. E., et al. (1975). "Phenylalanine hydroxylase activity in liver biopsies from hyperphenylalaninemia heterozygotes: deviation from proportionality with gene dosage." Pediatric research 9(8): 632-634.

[297] Feldman, D. E. and Frydman, J. (2000). "Protein folding in vivo: the importance of molecular chaperones." Current opinion in structural biology 10(1): 26-33.

[298] Daniele, A., Scala, I., et al. (2009). "Functional and structural characterization of novel mutations and genotype-phenotype correlation in 51 phenylalanine hydroxylase deficient families from Southern Italy." The FEBS journal 276(7): 2048-2059.

[299] Scriver, C. R. and Waters, P. J. (1999). "Monogenic traits are not simple: lessons from phenylketonuria." Trends in genetics : TIG 15(7): 267-272.

Chapter 1

Molecular Genetics and Impact of *In Vitro* Residual Phenylalanine Hydroxylase Activity on Tetrahydrobiopterin Responsiveness in Turkish PKU Population

Steven F. Dobrowolski[a,1], Caroline Heintz[b,1], Trent Miller[c], Clinton Ellingson[d], Clifford Ellingson[c], Işıl Özer[f], Gulden Gökçay[f], Tolunay Baykal[f], Beat Thöny[b,g,h], Mübeccel Demirkol[f,*], Nenad Blau[b,g,h,*]

[a] *Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT, USA*

[b] *University Children's Hospital, Division of Clinical Chemistry and Biochemistry, Zürich, Switzerland*

[c] *Idaho Technology, Salt Lake City, UT, USA*

[d] *Department of Medicine, Pennsylvania State University, College of Medicine, Hershey, PA, USA*

[e] *Case Western Reserve University, School of Medicine, Cleveland, OH, US*

[f] *Istanbul University, Istanbul Faculty of Medicine, Children's Hospital, Division of Nutrition and Metabolism, Istanbul, Turkey*

[g] *Zürich Center for Integrative Human Physiology (ZIHP), Zürich, Switzerland*

[h] *Pediatric Research Center (PRC), Zürich, Switzerland*

[1]The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint first authors.

Published in Molecular Genetics and Metabolism 2011, 102 (2): 116-21.

Abstract

Background: The prevalence of phenylalanine hydroxylase (PAH)-deficient phenylketonuria (PKU) in Turkey is high (1 in 6500 births), but data concerning the genotype distribution and impact of the genotype on tetrahydrobiopterin (BH_4) therapy are scarce.

Objective: To characterize the phenotypic and genotypic variability in the Turkish PKU population and to correlate it with physiological response to BH_4 challenge.

Methods: We genotyped 588 hyperphenylalaninemic patients and performed a BH_4 loading test (20 mg/kg body weight) in 462 patients. Residual PAH activity of mutant proteins was calculated from available *in vitro* expression data. Data were tabulated in the BIOPKU database (www.biopku.org).

Results: Eighty-eight mutations were observed, the most common missense mutations being the splice variant c.1066-11G>A (24.6%). Twenty novel mutations were detected (11 missense, 4 splice site, and 5 deletion/insertions). Two mutations were observed in 540/588 patients (91.8%) but in 9 patients atypical genotypes with > 2 mutations were found 8 with p.R155H *in cis* with another variant) and in 19 patients mutations were found in BH_4-metabolizing genes. The most common genotype was c.1066-11G>A/c.1066-11G>A (15.5%). Approximately 22% of patients responded to BH_4 challenge. A substantial *in vitro* residual activity (average > 25% of the wild type enzyme) was associated with response to BH_4. In homozygous genotypes ($n = 206$), both severity of the phenotype ($r = 0.83$) and residual PAH activity ($r = 0.85$) correlate with BH_4 responsiveness.

Conclusion: Together with the BH_4 challenge, these data enable the genotype-based classification of BH_4 responsiveness and document importance of residual PAH activity. This first report of a large-scale genotype assessment in a population of Turkish PKU patients also documents a high prevalence (47%) of the severe classic phenotype.

Introduction

Phenylketonuria (PKU; OMIM# 261600) is an autosomal recessive disorder associated with deficient hepatic phenylalanine hydroxylase (PAH) activity [1]. PAH converts phenylalanine (Phe) to tyrosine in the presence of the essential cofactor tetrahydrobiopterin (BH_4), molecular oxygen, and Fe^{2+}. BH_4 is synthesized from guanosine triphosphate (GTP) in a biosynthetic pathway including the enzymes GTP cyclohydrolase I (GTPCH; gene *GCH1*), 6-pyruvoyl-tetrahydropterin synthase (PTPS; gene *PTS*), and sepiapterin reductase (SR; gene *SPR*). The oxidized cofactor is regenerated in two enzymatic steps involving pterin-4a-carbinolamine dehydratase (PCD; gene *PCBD1*) and dihydropteridine reductase (DHPR; gene *QDPR*) [2]. Mutations in genes coding for PAH and BH_4-metabolizing enzymes result in hyperphenylalaninemia (HPA) [3]. *SPR* deficiency and autosomal dominant *GCH1* deficiency present without HPA [4]. BH_4 deficiencies are more severe than PKU, and in addition to HPA present with catecholamines and serotonin deficiency [5].

The overall prevalence of PKU in Europe and the United States is about 1 in 10,000 live births. Higher disease incidence is observed in cultures where consanguinity is practiced (e.g., Turkey, Saudi Arabia, or Gaza; ca. 1 in 3500 - 6500); however, in regions such as Finland the incidence is low (1 in > 100,000). Prevalence of BH_4 deficiencies is about 1 - 2% of all HPAs [6].

Late-diagnosed, untreated PKU leads to severe neurological impairment including mental retardation, microcephaly, autistic behavior, eczema, and seizures [7], particularly in the most severe forms of PAH deficiency, “classic PKU” (blood Phe concentrations > 1200 µmol/L). Less severe forms include mild PKU (blood Phe concentrations 600 - 1200 µmol/L) and mild HPA without any clinical findings (blood Phe concentrations < 600 µmol/L). Hyperphenylalaninemic patients are identified through prospective newborn screening and follow-on diagnostic procedures will identify the defective gene, enabling early initiation of appropriate therapy [8]. Not every HPA patient is routinely tested for DNA mutations.

The observation that serum Phe concentration may be controlled in a subset of PKU patients through oral administration of synthetic 6*R*-BH_4 [9] and reports of a relatively high incidence (20 - 30%) of BH_4 responsiveness [10, 11] provided an alternative to the traditional low Phe diet [14]. A number of studies documented that PAH-deficient patients with mild to moderate phenotypes are more likely to benefit from BH_4 therapy [10 - 14]. In some patients Phe concentration may be controlled with BH_4 monotherapy; however, others require a combination of BH_4 and dietary restrictions to maintain blood Phe in the therapeutic range

while increasing daily Phe tolerance [15 - 18]. Mechanisms of BH_4 responsiveness are multifactorial [19]. Current data suggest the most common mechanism by which BH_4 rescues PAH function is by acting as a pharmacological chaperone promoting proper enzyme folding, which in turn reduces enzyme degradation and inactivation [20, 21].

The *PAH*db (www.pahdb.mcgill.ca/) has cataloged over 500 mutations in the *PAH* gene [22], while the BIOPKU database (www.bh4.org/BH4DatabasesBiopku.asp) describes an approximately equal number of *PAH* genotypes and their association with BH_4 response [23]. A systematic review of PKU in Europe identified 29 mutations that may be regarded as prevalent in European populations [24], but there are very few reports on the molecular basis of PKU in Turkey [25, 26].

Herein are presented *PAH* genotypes of 588 Turkish PKU patients where 88 mutations were identified; among these are 20 novel mutations. Data from oral BH_4 challenge in 462 patients are reported. Comparisons are made relating BH_4 response with the genotype, residual *in vitro* PAH activity, and disease phenotype. The results extend the knowledge of the genotypic PKU variation in the Turkish PKU population and document a high prevalence of classical PKU (47%), a relatively high proportion (22%) of potential candidates for the BH_4 therapy, and the common occurrence of BH_4 deficiencies (2.4%) within this study.

Patients and methods

Patients and samples

A total of 588 hyperphenylalaninemic patients were investigated. At the time of diagnosis, 165 patients presented with mild HPA (blood Phe < 600 μmol/L), 130 with mild PKU (blood Phe 600 - 1200 μmol/L), and 274 patients presented with classic PKU (blood Phe > 1200 μmol/L). Nineteen patients with BH_4 deficiencies presented with a variable range of blood Phe (9 mild HPA, 7 mild PKU, 3 classic PKU). Forty-six percent of patients were the offspring of consanguineous mating (Table 1.1); however, an even higher percentage (48.7%) displayed mutation homozygosity, suggesting inbreeding (Suppl. Table 1).

Nine pedigrees, where > 2 mutations were identified, are included in this study. The majority of patients (~75%) were identified through prospective newborn screening, while the remainders were identified by selective screening. Blood specimens were collected on filter paper cards by finger or heel prick, and all tests were performed within routine clinical and biochemical investigation and in accordance with local regulations. Blood phenylalanine was measured using a fluorometric method until 2003, and tandem mass spectrometry was used afterwards. The first confirmatory quantitative phenylalanine was performed during

clinical assessment when the child was provided a normal diet. Informed consent for genotype assessment was obtained from all subjects. The University of Utah Institutional Review Board approved the plan to receive de-identified specimens for assessment of the *PAH* gene and genes of the BH_4 synthesis/recycling pathways.

Table 1.1: Consanguinity in Turkish PKU patients investigated in this study.

Related marriage	Number of families	%
No consanguinity	221	43.1
No consanguinity, but parents from same village	57	11.1
1st grade cousin	149	29.0
1.5 grade cousin	10	1.9
2 grade cousin	36	7.0
2.5 grade cousin	2	0.4
3 grade cousin	27	5.3
3.5 grade cousin	2	0.4
4 grade cousin	9	1.8

Loading test with BH_4

A single-dose BH_4 challenge (20 mg/kg body weight) was performed on 462 PKU patients (81%) (Schircks Laboratories, Switzerland). Three different protocols were used: A) Prior to 1999, a partially active formulation of BH_4, containing a mixture of the active R enantiomer and inactive S enantiomer (66.6% 6*R*-BH_4 and 33.3% 6*S*-BH_4), was used to challenge 166 patients. Thus a 20 mg tablet contained 13.3 mg of biologically active BH_4. In this subset, serum Phe was monitored over 8 h. B) A fully active formulation of BH_4 (6*R*-BH_4) was utilized post-1999. Among the 296 patients challenged, Phe was monitored over an 8-h period (0, 4, and 8 h) in 104 patients and C) over a 24-h period (0, 4, 8, and 24 h) in 192 patients. Data from patients whose plasma Phe concentration was monitored over 24 h were used for genotype-phenotype correlation and determination of residual PAH activity. In all BH_4 challenge protocols, response was defined as a sustained reduction of blood Phe concentration by ≥ 30% from the pre-challenge baseline [27].

Assessment of the PAH, PTS, and QDPR genes

DNA was prepared from dried blood on filter paper as previously described [28]. The PAH gene was assessed utilizing a previously described system involving high-resolution melt profiling and follow-on DNA sequencing of regions displaying aberrant melting profiles [29, 30]. DNA sequence data were analyzed using Mutation Surveyor software (Softgenetics, State College, PA, USA). The protocols utilized in assessment of PTS and QDPR also involved high-resolution melt profiling and follow-on DNA sequencing of regions displaying

aberrant melting profiles. The specifics of these assessments will be included in a separate study.

In several instances, PAH-deficient patients were identified with > 2 mutations in the *PAH* gene. When family participation could be recruited in such cases, blood samples were obtained from parents and other first-degree relatives for performance of pedigree studies to determine the *cis/trans* relationship between the mutations.

Relative residual PAH activity

Relative residual PAH activity ('PAH activity') was calculated from data provided from *in vitro* experiments using recombinantly expressed mutant proteins in eukaryotic cells. PAH activity is the average of the sum of activities of both alleles, and expressed as the percentage of the wild type enzyme. Expression data were compiled from the *PAH*db (www.pahdb.mcgill.ca/). Calculated *in vitro* 'PAH activity' is most probably different from *in vivo* enzyme activity. A splice site mutation is estimated as having no 'PAH activity' if it is associated with classic phenotype in > 95% of patients and is not recognized to facilitate a response to BH_4. Some splice site mutations may, however, produce wild type protein (albeit at a reduced level) and are thus associated with milder phenotypes.

Phenotype scoring

Phenotype scoring was utilized for patients with homozygous mutations. Phenotype severity was scored according to blood Phe levels assigning a score of 1 for the mildest HPA (lowest blood Phe levels) and a score of 10 for the severe classic PKU (highest blood Phe levels).

Statistical analysis

Statistical analysis was performed using WinSTAT 2007.1 for Excel (R. Fitch Software, Germany). Passing-Bablok regression analysis was used to compare the relative residual 'PAH activity' and phenotypes with BH_4 responsiveness.

Results

PAH genotypes

Among the patients genotyped two mutations were observed in 540/569 (94.9%) patients (Suppl. Table 1). A single mutation was observed in 29 patients (data not shown) and in no instances was there a PAH-deficient patient in which no mutation was observed. A total of 88 mutations were observed and Table 1.2 provides those mutations that were observed with ≥ 3% allele frequency.

Table 1.2: Most common mutations (AF>3%) found in Turkish patients with PKU.

Sequence variation	Effect	Alleles	Allele frequency (%)	Metabolic phenotype*	PAH activity** (%)
c.1066-11G>A	Splice dysfunction	273	24.6	cPKU	nd
c.782G>A	p.R261Q	96	8.7	mPKU	38.5
c.842C>T	p.P281L	93	8.4	cPKU	<1
c.143T>C	p.L48S	78	7.0	mPKU	39.0
c.1222C>T	p.R408W	71	6.4	cPKU	1.0
c.898G>T	p.A300S	56	5.0	MHP	31.0
c.1169A>G	p.E390G	46	4.1	MHP	72.5
c.441+5G>T	Splice dysfunction	33	3.0	cPKU	nd

MHP: mild HPA; mPKU: mild PKU; cPKU: classic PKU; nd: not determined.
* Based on phenotype characteristics of functionally hemizygous individuals (BIOPKUdb).
** Of the wild-type activity when recombinantly expressed in eukaryotic cell system [23].

The most frequently encountered *PAH* genotypes and their association with BH_4 responsiveness are shown in Table 1.3.

Table 1.3: Most common genotypes and association with BH_4 responsiveness.

PAH genotype		Frequency	Metabolic phenotype	PAH activity* (%)	Response to BH_4 challenge
Allele 1	Allele 2				
c.1066-11G>A	c.1066-11G>A	84 (15.5%)	cPKU	nd	−
p.P281L	p.P281L	27 (5.0%)	cPKU	<1	−
p.R261Q	p.R261Q	27 (5.0%)	mPKU	38.5	+
p.R408W	p.R408W	15 (2.8%)	cPKU	<1	−
p.L48S	c.1066-11G>A	13 (2.4%)	mPKU	nd	+/−
p.R408W	c.1066-11G>A	13 (2.4%)	cPKU	nd	−
p.L48S	p.L48S	12 (2.2%)	mPKU	39.0	+
c.441+5G>T	c.441+5G>T	11 (2.0%)	cPKU	nd	−
p.A300S	c.1066-11G>A	10 (1.8%)	MHP	nd	+
p.P281L	c.1066-11G>A	10 (1.8%)	cPKU	nd	−
p.A300S	p.A300S	9 (1.7%)	MHP	31.0	+
p.E390G	p.E390G	8 (1.5%)	MHP	42.5	+

MHP: mild HPA; mPKU: mild PKU; cPKU: classic PKU; nd: not determined.
* Of the wild-type activity when recombinantly expressed in eukaryotic cell system [23].

Consanguinity and inbreeding are apparent in that 8 of the 12 most frequently observed genotypes involve homozygosity and furthermore the c.1066-11G>A mutation is represented in 5 of the most common genotypes. Twenty novel mutations were identified, with p.Y204X occurring in 15 alleles (Table 1.4).

Table 1.4: Novel mutations detected in Turkish PKU patients.

PAH mutation	Nucleotide aberration	Location	Number of alleles
p.Q20H	c.60G>C	Exon 1	2
p.L37X	c.48-49insCT	Exon 2	3
p.F39del	c.113-115delTCT	Exon 2	1
p.E57K	c.169G>A	Exon 3	1
p.D75X	c.197-204del8	Exon 3	1
p.P119S	c.355C>T	Exon 4	1
p.W120fs	c.358delT	Exon 4	1
p.G148D	c.443G>A	Exon 5	2
p.Y204X	c.590-611del22	Exon 6	15
p.E280A	c.839A>C	Exon 7	7
p.P281R	c.842C>G	Exon 7	2
IVS7+4A>G	c.842+4A>G	Intron 7	2
p.L293S	c.878T>C	Exon 8	1
IVS9-7A>G	c.970-7A>G	Intron 9	2
IVS10-7C>A	c.1066-7C>A	Intron 10	1
p.F382L	c.1144T>C	Exon 11	2
p.K396R	c.1187A>G	Exon 11	2
p.V399A	c.1196T>C	Exon 11	2
IVS11-2A>G	c.1200-2A>G	Intron 11	1
p.Y417C	c.1250A>G	Exon 12	4

Table 1.5 shows that a high number of deletions (n = 54) and insertions (n = 5) were observed.

Table 1.5: Deletions and insertions found in Turkish PKU patients.

PAH variation	Effect	Alleles
c.1089delG	p.K363>Nfs	19
c.592_613del22	p.Y198Sfs	15
c.165delT	p.F55>Lfs	12
c.47_48delCT	p.S16>XfsX1	3
c.48_49insCT	p.L37X	3
c.266_267insC	p.P89>Pfs	2
c.1087_1088delAA	p.K363>Afs	2
c.197_204del8	p.D75X	1
c.358delT	p.F149X	1
c.113_115delTCT	p.F39del	1

Within the same group of genotypes, BH_4 responsiveness is equally distributed and frequently associated with mild HPA or mild PKU. In several instances, patients with 3 or even 4 mutations were identified (Table 1.6). Frequently the missense mutations p.R115H and p.A300S were observed together. To determine the inheritance phase of sequence

variants when > 2 mutations are observed, specimens from parents and other first-degree relatives were recruited. Pedigree studies demonstrate a relatively common compound mutation in Turkish PKU patients where p.R155H and p.A300S are present on a common chromosome. Although not all parents were tested for hyperphenylalaninemia, two mothers presented with mild HPA (blood Phe levels 170 - 255 μmol/L).

Table 1.6: Turkish PKU genotypes with additional mutations in *cis*.

Allele 1	Allele2	Mutations in *cis*	Metabolic phenotype	Number of patients
p.R176X	p.A300S	p.R155H	mPKU	2*
p.R155H	p.A300S	–	–	Mother
p.R176X	Wild-type	–	–	Father
IVS10-11G>A	IVS10-11G>A	p.L430P	cPKU	1
IVS10-11G>A	Wild-type	–	–	Mother
IVS10-11G>A	Wild-type	–	–	Father
p.P281L	p.P281L	p.R413P	cPKU	1
p.P281L	Wild-type	–	–	Mother
p.P281L	p.R413P	–	–	Father
p.L430P	p.A300S	p.R155H	MHP	1
p.R155H	p.A300S	–	–	Mother
p.L430P	Wild-type	–	–	Father
p.R413P	p.A300S	p.R155H	mPKU	1
p.R413P	Wild-type	–	–	Mother
p.R155H	p.A300S	–	–	Father
p.A300S	p.A300S	p.R155H	MHP	1
p.R155H	p.A300S	–	–	Father**
p.A300S	p.A300S	p.R155H/p.R155H	mPKU/cPKU	2
p.R155H	p.A300S	–	–	Mother
p.R155H	p.A300S	–	–	Father
p.R155H	p.R155H	p. R261Q	mPKU	1
p. R155H	Wild-type	–	–	Mother
p.R155H	p. R261Q	–	–	Father
IVS10-11G>A	IVS10-11G>A	p.R155H/ p.R155H	cPKU	1
p.R155H	IVS10-11G>A	–	–	Mother
p.R155H	IVS10-11G>A	–	–	Father

MHP: mild HPA; mPKU: mild PKU; cPKU: classic PKU.
* Siblings.
** Mother not tested.

Loading test with BH_4

Prior to 1999, a formulation of BH_4 containing 66.6% 6*R*-BH_4 and 33.3% 6*S*-BH_4 was utilized in challenge studies; thus, a 20 mg dose contained 13.3 mg of active drug. One hundred sixty-six patients (19% mild HPA, 19% mild PKU, 62% classic PKU) were challenged with 20 mg/kg (that in actuality provided 13.3 mg/kg), and Phe was monitored over 8 h. Among this group 6 patients (3.5%) responded with blood Phe reduction of ≥ 30% (Figure 1.1A). The residual 'PAH activity' of BH_4-responsive genotypes (4 mild HPA and 2 mild PKU) was between 25 and 72.5%. Additionally, three classic PKU patients responded to BH_4; however, their genotypes indicated that they had < 1% 'PAH activity' and as such were classified as BH_4 non-responders.

Two hundred ninety-six patients were challenged with the fully active BH_4 formulation; thus a dose of 20 mg/kg was achieved. One hundred four patients were monitored for 8 h post-challenge; among these, 19 patients were responsive including 11 mild HPA (31 - 73% 'PAH activity'), 4 mild PKU (38 - 39% 'PAH activity'), and 4 classic PKU (< 1% 'PAH activity') (Figure 1.1B). One hundred ninety-two patients, challenged with the fully active BH_4 formulation, were assessed for 24 h post challenge. Responsive patients included 45 mild HPA (16 - 56% PAH activity), 18 mild PKU (14 - 39% 'PAH activity'), and 4 classic PKU (< 1% 'PAH activity') (Figure 1.1C). Because the 8-h test may not identify some responsive patients, only data from the 24-h test were used for genotype–phenotype correlation.

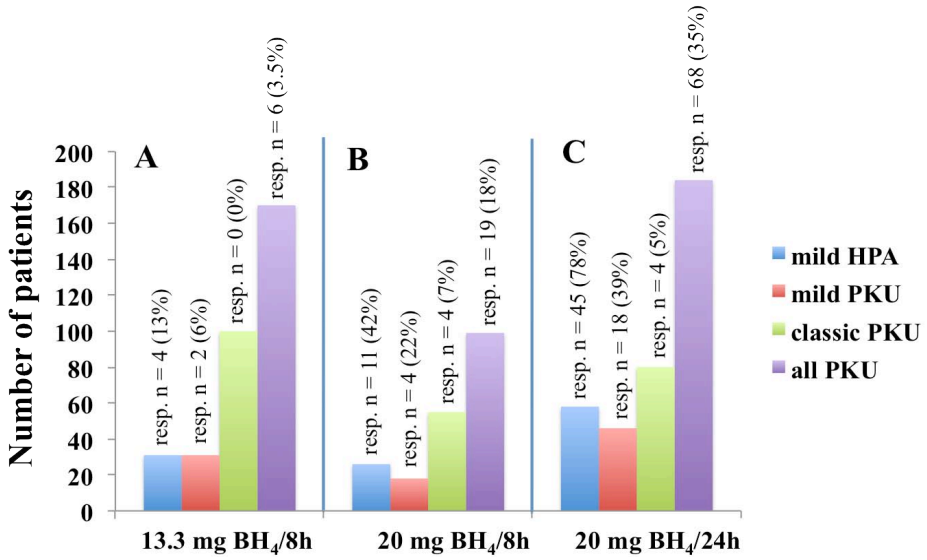


Figure 1.1: Number of patients loaded with (A) 13.3 mg BH_4 (old product) over 8 h; (B) 20 mg BH_4 over 8 h; and (C) 20 mg BH_4 over 24 h and numbers of responsive patients (blood Phe reduction by > 29.9%) defined by both the BH_4 challenge and genotype; resp. = BH_4-responder

BH_4 deficiency

Nineteen patients (8 female, 11 male) were diagnosed with BH_4 deficiency (11 with *QDPR* deficiency, 8 with *PTS* deficiency), all of them responding to BH_4 administration by lowering blood phenylalanine levels by more than 80% after 24 h (data not shown). These patients require a unique treatment regimen that includes neurotransmitter precursors and will be described in a separate study.

Phenotype, genotype, PAH activity, and BH_4 responsiveness

For the comparison between the genotype, phenotype, BH_4 responsiveness, and residual 'PAH activity,' the 10 most common homozygous genotypes (total n = 206) with a frequency of > 5 patients were compared, utilizing the severity of the phenotype and the residual 'PAH activity' calculated from *in vitro* experiments (Table 1.7).

Table 1.7: Ten most common homozygous genotypes occurring in more than 5 Turkish PKU patients with different phenotypes, residual *in vitro* 'PAH activity', percentage of responders, and calculated phenotype score. Phenotype score was calculated for genotypes presented with different phenotypes.

PAH genotype		Number of patients	PAH activity (%)*	BH_4 responder (%)	Phenotypes (%)			Phenotype score**
Allele 1	Allele 2				MHP	mPKU	cPKU	
p.R408W	p.R408W	15	2.0	0.0	0	7	93	9.7
IVS4+5G>T	IVS4+5G>T	11	<1	0.0	0	9	91	9.5
p.P281L	p.P281L	27	1.0	3.7	4	11	85	9.1
IVS10-11G>A	IVS10-11G>A	84	<1	3.6	1	18	81	9.0
p.A300S	p.A300S	9	31.0	25.0	0	22	78	8.9
p.R252W	p.R252W	7	1.0	20.0	29	29	43	6.0
p.R261Q	p.R261Q	27	38.5	39.1	11	67	22	5.7
p.L48S	p.L48S	12	39.0	44.4	42	33	25	4.6
p.R241C	p.R241C	6	25.0	40.0	50	33	17	3.8
p.E390G	p.E390G	8	72.5	40.0	100	0	0	1.0

MHP: mild HPA; mPKU: mild PKU; cPKU: classic PKU.
* Compared with the wild-type enzyme.
** Lowest score (1) for mild HPA and highest score (10) for classic PKU.

For most homozygous genotypes distribution of the phenotype was clear (e.g., p.R408W/p.R408W with no 'PAH activity,' classic phenotype, and 100% non-responsiveness). However, some genotypes (e.g., p.L48S/p.L48S) show a high inconsistency with regard to both phenotype (42% mild HPA, 33% mild PKU, 25% classic PKU) and BH_4 responsiveness (44.4% responder) (Suppl. Table 1).

Phenotype severity was scored according to highest blood Phe levels assigning a score of 1 for the mildest HPA and a score of 10 for the severe classic PKU. A good correlation ($r = 0.83$) was observed for BH_4 responsiveness and 'PAH activity' (Figure 1.2A) and with $r = 0.85$ for BH_4 responsiveness and phenotype (Figure 1.2B). Similar calculations with compound heterozygous genotypes yielded low correlation coefficients (data not shown).

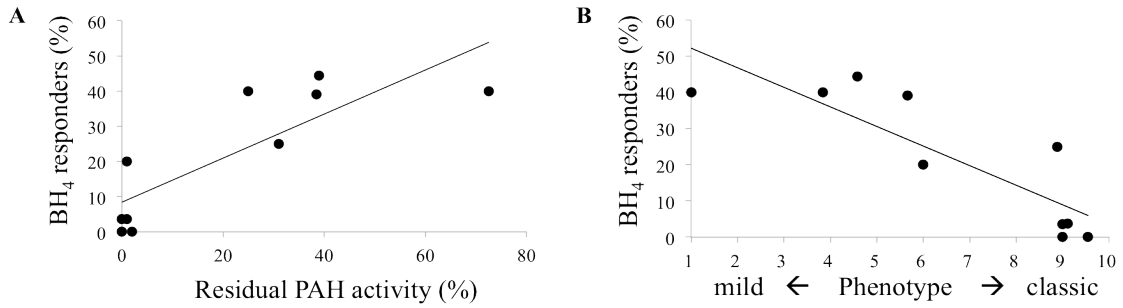


Figure 1.2: Correlation between (A) BH_4 responsiveness and residual 'PAH activity', and (B) BH_4 responsiveness and phenotype in patients with 10 most common ($n \geq 6$) homozygous mutations. Phenotype was scored according to the number of mild HPA, mild PKU or classic PKU patients within the same genotype and with the lowest score (1) for mild HPA and highest score (10) for classic PKU. For details see Table 1.7.

Discussion

The observed frequency of PAH-deficient PKU is higher in the Turkish population than in either Europe or the United States. Consanguinity, as a social norm in some communities within Turkish culture, has led to an increased frequency of PKU; furthermore, inbreeding within ethnic groups has also contributed to an increased disease frequency. An additional problem is delayed diagnosis and the quality of dietary management, both issues are a consequence of a paucity of PKU centers and the long distance patients must often travel to obtain care. The need for effective newborn screening and follow-on diagnostic procedures to categorize prospectively identified HPA newborns in the Turkish population is evident. We report a large-scale assessment of Turkish PKU patients and critically examine both PAH genotypes and response to challenge with BH_4.

Genotypes observed among the patient cohort showed homozygosity at a rate of ~ 48%. A high rate of homozygosity is not unique to Turkish PKU patients as we previously reported a high rate of homozygosity for the p.R408W mutation (43%) along with a limited spectrum of mutations among patients in western Poland [29]. As this study identified 88 different PAH mutations, including 20 novel mutations, the spectrum of PAH mutations in the Turkish population is relatively diverse. A unique genotypic feature of Turkish PKU patients is the number of in *cis* compound mutations (see Table 1.6). The p.R155H mutation was identified in *cis* with p.A300S in several patients. Upon reevaluation of a previously reported patient, we found p.R115H to be in *cis* with p.D143G (data not shown) [31]. Previously we determined that p.R155H is a mild mutation with minimal impact upon characteristics of the PAH enzyme; however, the combined influence of two missense changes in the same polypeptide chain could impact the enzyme more so than either mutation individually [31].

The mutation spectrum in Turkish PKU patients reveals c.1066-11G>A (24.6%), p.R261Q (8.7%) and p.P281L (8.4%) to be frequently associated with mild to classic PKU. This finding is in accordance with previous reports from small studies involving 44 Turkish PKU patients [25, 26] and patients of Turkish origin in Germany [32]. Four of the 10 most common mutations (p.R261Q, p.L48S, p.A300S, and p.E390G) present with substantial residual activity (31 - 72.5% of the wild type PAH) when expressed in eukaryotic cell systems, and are associated with mild HPA or mild PKU. It has been suggested that p.E390G has only a modestly deleterious impact on the PAH enzyme [26] and the same may apply to the p.A300S. In contrast, p.L48S with 39% residual 'PAH activity' may be associated with both mild and classic PKU [23, 33]; thus, the mutation should be classified as equivocal in

regard to being BH_4-responsive. However, classifying alleles as BH_4-responsive or non-responsive has limited utility, particularly when compound heterozygosity is involved. Interallelic complementation between unique PAH missense enzymes may exert a dominant negative effect in regard to BH_4 response. Also, *in vitro* expressed activity may not necessarily represent the PAH activity in hepatocytes. Bartholomé et al. [34] showed that patients with classic PKU had no PAH activity in liver needle biopsies, patients with mild to moderate PKU showed up to 6% residual activity, and patients with mild HPA showed 8 - 35% of the normal activity. This and other studies [35, 36] have shown that HPA occurs at *in vivo* PAH activities below 10 - 15% and that residual activity is essential for maintaining normal hepatic phenylalanine homeostasis. Thus, *in vitro* data should be interpreted cautiously, particularly with regard to BH_4 responsiveness. As liver needle biopsy is no longer justified in PKU patients, *in vitro* assessment of mutant PAH proteins is the primary source of information concerning residual enzyme activity, which may be applied to patient phenotypes.

Our study documents, in contrast to a previous report [23], that some mutations (e.g., p.R158Q) with < 20% 'PAH activity' should not be classified as BH_4-responsive. Alternatively, some splice site variants (e.g., c.1066-3C>T) are clearly associated with response to BH_4. It is possible that a BH_4-responsive splicing mutation may not be fully penetrant and the gene may produce multiple mRNAs, including some wild type *PAH*-mRNA message. This hypothesis would explain a mild phenotype and BH_4 responsiveness in one patient from our cohort who is homozygous for c.1066-3C>T (Suppl. Table 1).

There is a single report on BH_4 response among 20 Turkish PKU patients (4 mild HPA, 16 mild to moderate PKU, no classic PKU) that estimates the prevalence of responsive patients to be 45%. The authors conclude that predicting BH_4 response based solely on the genotype is difficult owing to a small sample size and compound heterozygous genotypes [37]. In our study, we compared genotypes in which the residual 'PAH activity' of the mutated proteins was previously investigated with the outcome of the BH_4 test. As expected and reported in previous studies [11, 14], mild HPA and mild to moderate PKU patients are most likely to elicit a physiological response to BH_4.

For the first time, we are able to compare a large number of homozygous genotypes with the outcome of the BH_4 loading test and with the residual 'PAH activity.' Genotypes that lack residual 'PAH activity' (e.g., p.R408W/p.R408W or IVS4+5G>T/IVS4+5G>T) can be considered non-responsive to BH_4, eliminating the need for a clinical BH_4 challenge. Calculating the residual 'PAH activity' from the information available from *in vitro*

experiments may be useful for the prediction and/or exclusion of potential candidates for BH_4 therapy. This method is demonstrably more powerful than calculations based on a single mutation only.

Competing interests

None

Acknowledgments

This work is supported by the Swiss National Science Foundation grant no. 3100A0-119982/1 (to N.B. and B.T.) and by the American National Institutes of Health grant no. R44HD075156 (to S.F.D.).

References

[1] C.R. Scriver, S. Kaufman, Hyperphenylalaninemia: phenylalanine hydroxylase deficiency, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, B. Vogelstein (Eds.), The metabolic and molecular bases of inherited disease, McGraw-Hill, New York, 2001, pp. 1667–1724.

[2] B. Thöny, G. Auerbach, N. Blau, Tetrahydrobiopterin biosynthesis, regeneration, and functions, Biochem J 347 (2000) 1–26.

[3] B. Thöny, N. Blau, Mutations in the BH4-metabolizing genes GTP cyclohydroalse I, 6pyruvoyl-tetrahydropterin synthase, sepiapterin reductase, carbinolamine-4a dehydratase, and dihydropteridine reductase genes, Hum Mutat 27 (2006) 870–878.

[4] N. Blau, L. Bonafé, B. Thöny, Tetrahydrobiopterin deficiencies without hyperphenylalaninemia: diagnosis and genetics of Dopa-responsive dystonia and sepiapterin reductase deficiency, Mol Genet Metab 74 (2001) 172–185.

[5] N. Blau, B. Thöny, R.G.H. Cotton, K. Hyland, Disorders of tetrahydrobiopterin and related biogenic amines, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, B. Vogelstein (Eds.), The metabolic and molecular bases of inherited disease, McGraw-Hill, New York, 2001, pp. 1725–1776.

[6] I. Smith, B. Cook, M. Beasley, Review of neonatal screening programme for phenylketonuria, BMJ 303 (1991) 333–335.

[7] N. Blau, F.J. Van Spronsen, H.L. Levy, Phenylketonuria, Lancet 376 (2010) 1417–1427.

[8] F. Feillet, F.J. van Spronsen, A. MacDonald, F.K. Trefz, M. Demirkol, M. Giovannini, A. Bélanger-Quintana, N. Blau, Challenges and pitfalls in the management of phenylketonuria, Pediatrics 126 (2010) 333–341.

[9] S. Kure, D.C. Hou, T. Ohura, H. Iwamoto, S. Suzuki, N. Sugiyama, O. Sakamoto, K. Fujii, Y. Matsubara, K. Narisawa, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, J Pediatr 135 (1999) 375–378.

[10] C. Bernegger, N. Blau, High frequency of tetrahydrobiopterin-responsiveness among hyperphenylalaninemias: a study of 1919 patients observed from 1988 to 2002, Mol Genet Metab 77 (2002) 304–313.

[11] B. Fiege, N. Blau, Assessment of tetrahydrobiopterin (BH4)-responsiveness in phenylketonuria, J Pediatr 150 (2007) 627–630.

[12] A.C. Muntau, W. Röschinger, M. Habich, H. Demmelmair, B. Hoffmann, C.P. Sommerhoff, A.A. Roscher, Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria, N Engl J Med 347 (2002) 2122–2132.

[13] H. Levy, A. Milanowski, A. Chakrapani, M. Cleary, P. Lee, F.K. Trefz, C.B. Whitley, F. Feillet, A.S. Feigenbaum, J.D. Bebchuk, H. Christ-Schmidt, A. Dorenbaum, Efficacy of sapropterin dihydrochloride (tetrahydrobiopterin, 6R-BH4) for reduction of phenylalanine concentration in patients with phenylketonuria: a phase III randomized placebo-controlled study, Lancet 370 (2007) 504–510.

[14] B.K. Burton, D.K. Grange, A. Milanowski, G. Vockley, F. Feillet, E.A. Crombez, V. Abadie, C.O. Harding, S. Cederbaum, D. Dobbelaere, A. Smith, A. Dorenbaum, The response of patients with phenylketonuria and elevated serum phenylalanine to treatment with oral sapropterin dihydrochloride (6R-tetrahydrobiopterin): a phase II, multicentre, open-label, screening, J Inherit Metab Dis 30 (2007) 700–707.

[15] J.B. Hennermann, C. Bührer, N. Blau, B. Vetter, E. Mönch, Long-term treatment with tetrahydrobiopterin increases phenylalanine tolerance in children with severe phenotype of phenylketonuria, Mol Genet Metab 86 (2005) 86–90.

[16] A. Burlina, N. Blau, Effect of BH4 supplementation on phenylalanine tolerance, J Inherit Metab Dis 32 (2009) 40–54.

[17] A. Bélanger-Quintana, M.J. Garcia, M. Castro, L.R. Desviat, B. Perez, B. Mejia, M. Ugarte, M. Martinez-Pardo, Spanish BH4-responsive phenylalanine hydroxylase-deficient patients: evolution of seven patients on long-term treatment with tetrahydrobiopterin, Mol Genet Metab 86 (2005) 61–66.

[18] H. Shintaku, S. Kure, T. Ohura, Y. Okano, M. Ohwada, N. Sugiyama, N. Sakura, I. Yoshida, M. Yoshino, Y. Matsubara, K. Suzuki, K. Aoki, T. Kitagawa, Long-term treatment and diagnosis of tetrahydrobiopterin-responsive hyperphenylalaninemia with a mutant phenylalanine hydroxylase gene, Pediatr Res 55 (2004) 425–430.

[19] N. Blau, H. Erlandsen, The metabolic and molecular bases of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, Mol Genet Metab 82 (2004) 101–111.

[20] A.L. Pey, A. Martinez, The activity of wild-type and mutant phenylalanine hydroxylase and its regulation by phenylalanine and tetrahydrobiopterin at physiological and pathological concentrations: an isothermal titration calorimetry study, Mol Genet Metab 86 (2005) 43–53.

[21] S.W. Gersting, K.F. Kemter, M. Staudigl, D.D. Messing, M.K. Danecka, F.B. Lagler, C.P. Sommerhoff, A.A. Roscher, A.C. Muntau, Loss of function in phenylketonuria is caused by impaired molecular motions and conformational instability, Am J Hum Genet 83 (2008) 5–17.

[22] C.R. Scriver, M. Hurtubise, D. Konecki, M. Phommarinh, L. Prevost, H. Erlandsen, R. Stevens, P.J. Waters, S. Ryan, D. McDonald, C. Sarkissian, PAHdb 2003: what a locus-specific knowledgebase can do, Hum Mutat 21 (2003) 333–344.

[23] M.R. Zurflüh, J. Zschocke, M. Lindner, F. Feillet, C. Chery, A. Burlina, R. Stevens, B. Thöny, N. Blau, Molecular genetics of tetrahydrobiopterin responsive phenylalanine hydroxylase deficiency, Hum Mutat (2008) 167–175.

[24] J. Zschocke, Phenylketonuria mutations in Europe, Hum Mutat 21 (2003) 345–356.

[25] M. Özgüc, I. Özalp, T. Coskun, E. Yilmaz, H. Erdem, S. Ayter, Mutation analysis in Turkish phenylketonuria patients, J Med Genet 30 (1993) 129–130.

[26] E. Yilmaz, F. Cali, V. Roman, I. Özalp, T. Coskun, A. Tokatli, H.S. Kalkanoglu, M. Özgüc, Molecular basis of mild hyperphenylalaninaemia in Turkey, J Inherit Metab Dis 23 (2000) 523–525.

[27] N. Blau, A. Belanger-Quintana, M. Demirkol, F. Feillet, M. Giovannini, A. MacDonald, F.K. Trefz, F.J. van Spronsen, Optimizing the use of sapropterin (BH4) in the management of phenylketonuria, Mol Genet Metab 96 (2009) 158–163.

[28] E.M. Heath, D.P. O'Brien, R. Banas, E.W. Naylor, S. Dobrowolski, Optimization of an automated DNA purification protocol for neonatal screening, Arch Pathol Lab Med 123 (1999) 1154–1160.

[29] S.F. Dobrowolski, K. Borski, C.C. Ellingson, R. Koch, H.L. Levy, E.W. Naylor, A limited spectrum of phenylalanine hydroxylase mutations is observed in phenylketonuria patients in western Poland and implications for treatment with 6R-tetrahydrobiopterin, J Hum Genet 54 (2009) 335–339.

[30] S.F. Dobrowolski, C. Ellingson, T. Coyne, J. Grey, R. Martin, E.W. Naylor, R. Koch, H.L. Levy, Mutations in the phenylalanine hydroxylase gene identified in 95 patients

with phenylketonuria using novel systems of mutation scanning and specific genotyping based upon thermal melt profiles, Mol Genet Metab 91 (2007) 218–227.

[31] S.F. Dobrowolski, A.L. Pey, R. Koch, H. Levy, C.C. Ellingson, E.W. Naylor, A. Martinez, Biochemical characterization of mutant phenylalanine hydroxylase enzymes and correlation with clinical presentation in hyperphenylalaninaemic patients, J Inherit Metab Dis 32 (2009) 10–21.

[32] J. Zschocke, G.F. Hoffmann, Phenylketonuria mutations in Germany, Hum Genet 104 (1999) 390–398.

[33] F.K. Trefz, D. Scheible, H. Gotz, G. Frauendienst-Egger, Significance of genotype in tetrahydrobiopterin-responsive phenylketonuria, J Inherit Metab Dis 32 (2009) 22–26.

[34] K. Bartholomé, P. Lutz, H. Bickel, Determination of phenylalanine hydroxylase activity in patients with phenylketonuria and hyperphenylalaninemia, Pediatr Res 9 (1975) 899.

[35] P. Justice, M.E. O'Flynn, D.Y. Hsia, Phenylalanine hydroxylase activity in hyperphenylalaninaemia, Lancet 1 (1967) 928–929.

[36] H.K. Berry, M.H. Hsieh, M.K. Bofinger, W.K. Schubert, Diagnosis of phenylalanine hydroxylase deficiency (phenylketonuria), Am J Dis Child 136 (1982) 111–114.

[37] S. Yildirim, A. Tokatli, E. Yilmaz, T. Coskun, Assessment of tetrahydrobiopterin responsiveness in Turkish hyperphenylalaninemic patients, Turk J Pediatr 49 (2007) 1–6.

Supplementary Table 1. Summary of all PKU patients with a full genotype and investigated for BH_4 responsiveness.

Allele 1	Allele 2	No of patients	GF (%)	mHPA	mPKU	cPKU	Homozygous	Resp @24h	Non-resp @24h	Resp @8h	Non-resp @8h	Not tested	Total resp	Total non-resp	%Resp	PAH act.
IVS10-11G>A	IVS10-11G>A	84	15.5	1	15	68	84	2	30	1	51	0	3	81	3.6	0.0
p.P281L	p.P281L	27	5.0	1	3	23	27	0	11	1	15	0	1	26	3.7	1.0
p.R261Q	p.R261Q	27	5.0	3	18	6	27	7	6	2	8	4	9	14	39.1	38.5
p.R408W	p.R408W	15	2.8	0	3	12	15	0	3	0	5	4	0	8	0	2.0
p.L48S	IVS10-11G>A	13	2.4	1	5	7	-	2	3	0	7	1	2	10	16.7	19.5
p.R408W	IVS10-11G>A	13	2.4	1	4	8	-	0	4	0	4	5	0	8	0.0	1.0
p.L48S	p.L48S	12	2.2	5	4	3	12	3	3	1	2	3	4	5	44.4	39.0
IVS4+5G>T	IVS4+5G>T	11	2.0	0	1	10	11	0	3	0	7	1	0	10	0	0.0
p.A300S	IVS10-11G>A	11	2.0	11	0	0	-	6	0	2	1	2	8	1	88.9	15.5
p.P281L	IVS10-11G>A	10	1.8	0	2	8	-	1	1	1	4	4	2	5	28.6	0.5
p.A300S	p.A300S	9	1.7	0	2	7	9	2	2	0	4	1	2	6	25.0	31.0
p.E390G	p.E390G	8	1.5	8	0	0	8	0	0	2	3	3	2	3	40.0	72.5
p.L48S	p.P281L	8	1.5	2	4	2	-	0	3	0	3	2	0	6	0	20.0
p.R252W	p.R252W	7	1.3	2	2	3	7	0	2	1	2	2	1	4	20.0	1.0
p.R241C	p.R241C	6	1.1	3	2	1	6	1	0	1	3	0	2	3	40.0	25.0
p.R261Q	p.A300S	6	1.1	6	0	0	-	1	1	0	0	4	1	1	50.0	37.0
p.R261Q	IVS10-11G>A	6	1.1	0	2	4	-	0	2	0	2	2	0	4	0	19.0
p.K363>Nfs	p.K363>Nfs	5	0.9	0	1	4	5	0	1	0	3	1	0	4	0	nd
p.L48S	p.R261Q	5	0.9	0	2	3	-	0	1	0	4	0	0	5	0	39.0
p.R241C	p.R408W	5	0.9	0	4	1	-	2	0	0	2	1	2	2	50.0	13.5
p.Y204X	p.Y204X	5	0.9	0	0	5	5	0	2	0	3	0	0	5	0	0
p.P211T	IVS10-11G>A	4	0.7	3	1	0	-	1	2	0	1	0	1	3	25.0	nd
p.L48S	p.E390G	3	0.6	2	1	0	-	2	0	0	0	1	2	0	100	55.8
p.A403V	IVS4+5G>T	3	0.6	3	0	0	-	0	0	0	0	0	0	0	-	nd

Supplementary Table 1 continuing

Allele 1	Allele 2	No of genotypes	GF (%)	mHPA	mPKU	cPKU	Homozygous	Resp @24h	Non-resp @24h	Resp @8h	Non-resp @8h	Not tested	Total resp	Total non-resp	%Resp	PAH act.
p.A403V	IVS10-11G>A	3	0.6	3	0	0	-	0	0	0	1	2	0	1	0.0	nd
p.E178G	IVS10-11G>A	3	0.6	2	1	0	-	1	0	0	2	0	1	2	33.3	nd
p.E280A	p.E280A	3	0.6	0	1	2	3	0	1	0	1	1	0	2	0	nd
p.E390G	IVS10-11G>A	3	0.6	3	0	0	-	1	0	0	0	2	1	0	100	36.3
p.F55>Lfs	p.P281L	3	0.6	0	0	3	-	0	0	1	2	0	1	2	33.3	0.5
p.L48S	p.R408W	3	0.6	1	1	1	-	0	1	0	1	1	0	2	0	20.5
p.L48S	p.R261X	3	0.6	1	1	1	-	0	0	0	2	1	0	2	0	20
p.P281L	p.R408W	3	0.6	0	1	2	-	0	1	0	2	0	0	3	0	1.5
p.R158Q	IVS10-11G>A	3	0.6	0	0	3	-	0	2	0	1	0	0	3	0	5
p.R243Q	p.R243Q	3	0.6	0	0	3	3	0	0	0	3	0	0	3	0	23
p.R243X	p.R243X	3	0.6	0	1	2	3	0	0	0	1	2	0	1	0	1
p.R261Q	p.E390G	3	0.6	3	0	0	-	2	0	1	0	0	3	0	100	55.5
p.R261Q	p.A403V	3	0.6	3	0	0	-	0	0	0	0	3	0	0	-	35.3
IVS2+5G>C	IVS2+5G>C	2	0.4	0	0	2	2	0	0	1	0	1	1	0	100	0
IVS7+1G>A	IVS7+1G>A	2	0.4	1	1	0	2	0	0	0	0	0	0	0	-	nd
p.A300S	IVS7+4A>G	2	0.4	2	0	0	-	1	0	0	0	1	1	0	100	nd
p.E178G	IVS2+5G>C	2	0.4	2	0	0	-	2	0	0	0	0	2	0	100	19.5
p.E280K	p.R408W	2	0.4	1	1	0	-	0	0	0	0	0	0	0	-	1.5
p.E390G	p.A403V	2	0.4	2	0	0	-	0	0	1	0	1	1	0	100	52.3
p.F55>Lfs	p.E390G	2	0.4	1	1	0	-	1	0	0	1	0	1	1	50	nd
p.F55>Lfs	IVS10-11G>A	2	0.4	0	1	1	-	0	0	0	1	1	0	1	0	nd
p.K363>Nfs	IVS10-11G>A	2	0.4	0	1	1	-	0	2	0	0	0	0	2	0	nd
p.K396R	IVS10-11G>A	2	0.4	1	1	0	-	0	0	0	1	1	0	1	0	nd
p.L37X	p.R243X	2	0.4	0	0	2	-	0	0	1	1	0	1	1	50.0	0
p.L48S	p.F55>Lfs	2	0.4	0	1	1	-	0	0	0	1	1	0	1	0	nd

Supplementary Table 1 continuing

Allele 1	Allele 2	No of genotypes	GF (%)	mHPA	mPKU	cPKU	Homozygous	Resp @24h	Non-resp @24h	Resp @8h	Non-resp @8h	Not tested	Total resp	Total non-resp	%Resp	PAH act.
p.L48S	p.A403V	2	0.4	2	0	0	-	0	0	0	1	1	0	1	0	35.5
p.L48S	p.A300S	2	0.4	2	0	0	-	0	0	0	2	0	0	2	0	35
p.L48S	IVS4+5G>T	2	0.4	0	0	2	-	0	2	0	0	0	0	2	0	19.5
p.L48S	IVS2+5G>C	2	0.4	0	2	0	-	0	0	0	1	1	0	1	0	19.5
p.L48S	p.R241H	2	0.4	2	0	0	-	0	0	1	1	0	1	1	50.0	31
p.P281L	p.T323del	2	0.4	0	1	1	-	0	0	0	2	0	0	2	0	0.5
p.P281L	p.E390G	2	0.4	2	0	0	-	0	0	0	0	2	0	0	-	36.8
p.P89>Pfs	p.P281R	2	0.4	0	0	2	-	0	2	0	0	0	0	2	0	nd
p.R158Q	p.R261X	2	0.4	0	1	1	-	0	0	0	1	1	0	1	0	5.5
p.R158Q	p.R158Q	2	0.4	0	0	2	2	0	1	0	0	1	0	1	0	10
p.R176X	p.A300S	2	0.4	0	0	2	-	0	0	0	2	0	0	2	0	15.5
p.R241H	p.R241H	2	0.4	0	1	1	2	0	0	0	2	0	0	2	0	23
p.R243X	p.R261Q	2	0.4	0	1	1	-	0	0	0	1	1	0	1	0	19.8
p.R243X	p.K363>Nfs	2	0.4	0	1	1	-	0	0	0	1	1	0	1	0	nd
p.R243X	IVS10-11G>A	2	0.4	0	1	1	-	0	0	0	2	0	0	2	0	0
p.R261Q	p.P281L	2	0.4	0	1	1	-	0	0	0	1	1	0	1	0	19.8
p.T380M	IVS10-11G>A	2	0.4	2	0	0	-	0	0	0	2	0	0	2	0	nd
p.V230I	IVS10-11G>A	2	0.4	2	0	0	-	0	0	1	1	0	1	1	50.0	31.5
p.V388M	p.V388M	2	0.4	0	2	0	2	2	0	0	0	0	2	0	100	27.5
p.Y417C	p.Y417C	2	0.4	2	0	0	2	0	0	0	1	1	0	1	0	0
IVS10-3C>T	IVS10-3C>T	1	0.2	0	1	0	1	0	0	0	1	0	0	1	0	0
IVS10-7 C>A	IVS11-2A>G	1	0.2	0	0	1	-	0	1	0	0	0	0	1	0	0
IVS11+1G>C	IVS11+1G>C	1	0.2	0	0	1	1	0	0	0	1	0	0	1	0	0
IVS2-13T>G	IVS2-13T>G	1	0.2	0	0	1	1	0	0	0	1	0	0	1	0	0
IVS2-13T>G	IVS10-11G>A	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	0

Supplementary Table 1 continuing

Allele 1	Allele 2	No of genotypes	GF (%)	mHPA	mPKU	cPKU	Homozygous	Resp @24h	Non-resp @24h	Resp @8h	Non-resp @8h	Not tested	Total resp	Total non-resp	%Resp	PAH act.
IVS2+5G>C	IVS10-11G>A	1	0.2	0	0	1	-	1	0	0	0	0	1	0	100	0
IVS3-1G>A	IVS3-1G>A	1	0.2	0	0	1	1	0	0	0	1	0	0	1	0	0
IVS3+1G>A	IVS3+1G>A	1	0.2	0	0	1	1	0	1	0	0	0	0	1	0	0
IVS4-5C>G	IVS4-5C>G	1	0.2	0	0	1	1	0	0	0	1	0	0	1	0	0
IVS4+5G>T	IVS10-11G>A	1	0.2	0	0	1	-	0	1	0	0	0	0	1	0	0
IVS4+6T>C	IVS4+6T>C	1	0.2	0	0	1	1	0	1	0	0	0	0	1	0	0
IVS7+1G>A	IVS10-11G>A	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	0
IVS9-1G>T	IVS9-1G>T	1	0.2	0	0	1	1	0	1	0	0	0	0	1	0	0
IVS9-7A>G	IVS9-7A>G	1	0.2	0	0	1	1	0	0	0	1	0	0	1	0	0
p.A300S	p.T380M	1	0.2	1	0	0	-	0	0	0	1	1	0	1	0	nd
p.A300S	p.R413P	1	0.2	0	1	0	-	0	1	0	0	0	0	1	0	nd
p.A300S	p.R408W	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	16.5
p.A300S	p.L430P	1	0.2	1	0	0	-	0	1	0	0	0	0	1	0	nd
p.A300S	p.E390G	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	51.8
p.A300S	p.A403V	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	31.5
p.A300S	IVS4+5G>T	1	0.2	0	1	0	-	1	0	0	0	0	1	0	100	15.5
p.A300S	IVS11+1G>C	1	0.2	1	0		-	0	0	0	0	1	0	0	-	15.5
p.A403V	p.Y204X	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	16
p.A403V	IVS10-3C>T	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	16
p.D143G	p.R408W	1	0.2	0	1	0	-	0	1	0	0	0	0	1	0	nd
p.D222V	p.R243X	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	nd
p.D222V	p.R241H	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	nd
p.D338Y	p.D338Y	1	0.2	1	0	0	1	1	0	0	0	0	1	0	100	nd
p.D394H	p.D394H	1	0.2	1	0	0	1	0	0	1	0	0	1	0	100	nd
p.D394H	IVS10-11G>A	1	0.2	0	1	0	-	0	0	0	1	0	0	1	0	nd

Supplementary Table 1 continuing

Allele 1	Allele 2	No of genotypes	GF (%)	mHPA	mPKU	cPKU	Homozygous	Resp @24h	Non-resp @24h	Resp @8h	Non-resp @8h	Not tested	Total resp	Total non-resp	%Resp	PAH act.
p.D75X	p.A300S	1	0.2	1	0	0	-	0	0	1	0	0	1	0	100	15.5
p.E178G	p.V399A	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	nd
p.E178G	p.R261Q	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	38.8
p.E178G	p.E390G	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	55.8
p.E221G	p.E221G	1	0.2	1	0	0	1	1	0	0	0	0	1	0	100	nd
p.E280A	p.E390G	1	0.2	0	1	0	-	0	0	0	1	0	0	1	0	nd
p.E390G	p.Y204X	1	0.2	0	1	0	-	0	0	0	1	0	0	1	0	36.3
p.E390G	p.R408W	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	37
p.E390G	p.A434D	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	nd
p.E390G	IVS4+5G>T	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	36.3
p.E390G	IVS2+1G>A	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	36.3
p.E56D	p.Y356X	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	nd
p.E56D	p.R408W	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	nd
p.E57K	p.V388M	1	0.2	1	0	0	-	0	0	1	0	0	1	0	100	nd
p.F149X	p.P281L	1	0.2	0	1	0	-	0	0	0	0	1	0	0	-	0.5
p.F382L	p.F382L	1	0.2	1	0	0	1	0	0	0	0	1	0	0	-	nd
p.F39del	p.A300S	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	nd
p.F55>Lfs	p.R261Q	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	19.3
p.F55>Lfs	p.E178G	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	19.5
p.F55L	IVS2+5G>C	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	nd
p.I224T	p.A300S	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	nd
p.I224T	p.R408Q	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	nd
p.I65S	p.H170Q	1	0.2	0	1	0	-	0	0	0	0	1	0	0	-	nd
p.I65T	p.S350Y	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	nd
p.I65T	IVS10-11G>A	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	13

Supplementary Table 1 continuing

Allele 1	Allele 2	No of genotypes	GF (%)	mHPA	mPKU	cPKU	Homozygous	Resp @24h	Non-resp @24h	Resp @8h	Non-resp @8h	Not tested	Total resp	Total non-resp	%Resp	PAH act.
p.K363>Afs	p.K363>Afs	1	0.2	0	0	1	1	0	0	0	1	0	0	1	0	nd
p.K363>Nfs	IVS4+5G>T	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	nd
p.K363>Nfs	IVS2+5G>C	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	nd
p.L213P	p.E390G	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	nd
p.L213P	p.D415N	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	nd
p.L242F	IVS10-11G>A	1	0.2	0	1	0	-	0	0	0	0	1	0	0	-	nd
p.L333F	p.E390G	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	40
p.L347F	p.L347F	1	0.2	0	0	1	1	0	0	0	0	1	0	0	-	nd
p.L367Pfs	IVS10-11G>A	1	0.2	0	0	1	-	0	1	0	0	0	0	1	0	0
p.L37X	p.R261Q	1	0.2	0	1	0	-	0	1	0	0	0	0	1	0	20
p.L41F	IVS10-11G>A	1	0.2	0	1	0	-	0	0	0	1	0	0	1	0	5
p.L48S	p.Y387H	1	0.2	0	1	0	-	0	0	0	1	0	0	1	0	nd
p.L48S	p.V230I	1	0.2	0	1	0	-	0	0	0	1	0	0	1	0	46
p.L48S	p.T323del	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	nd
p.L48S	p.R252W	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	20
p.L48S	p.E178G	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	39
p.L48S	p.T328A	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	19.5
p.L48S	p.R261X	1	0.2	0	1	0	-	0	0	0	0	1	0	0	-	20
p.P119S	p.R261Q	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	nd
p.P211T	p.R408W	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	55.5
p.P211T	p.R261Q	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	55.5
p.P211T	p.A403V	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	55.5
p.P225T	p.P225T	1	0.2	0	0	1	1	0	1	0	0	0	0	1	0	nd
p.P279C	p.R408W	1	0.2	0	1	0	-	1	0	0	0	0	1	0	100	nd
p.P281L	p.V399A	1	0.2	0	1	0	-	0	0	0	0	1	0	0	-	nd

Supplementary Table 1 continuing

Allele 1	Allele 2	No of genotypes	GF (%)	mHPA	mPKU	cPKU	Homozygous	Resp @24h	Non-resp @24h	Resp @8h	Non-resp @8h	Not tested	Total resp	Total non-resp	%Resp	PAH act.
p.P281L	p.P366H	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	nd
p.P281L	p.D415N	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	47
p.P281L	p.A403V	1	0.2	1		0	-	1	0	0	0	0	1	0	100	16.5
p.P281L	IVS4-5C>G	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	nd
p.Q20H	p.Q20H	1	0.2	1	0	0	1	0	0	0	0	1	0	0	-	nd
p.R111X	p.R111X	1	0.2	0	0	1	1	0	0	0	1	0	0	1	0	0
p.R111X	p.P281L	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	1
p.R155H	p.R155H	1	0.2	1	0	0	1	0	0	0	0	1	0	0	-	nd
p.R158Q	p.R408W	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	6
p.R158Q	p.E390G	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	41.3
p.R169H	IVS10-11G>A	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	nd
p.R176X	p.R176X	1	0.2	0	0	1	1	0	0	0	1	0	0	1	0	1
p.R241C	p.R261X	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	13
p.R241C	p.L430P	1	0.2	1	0	0	-	0	1	0	0	0	0	1	0	nd
p.R241C	IVS10-11G>A	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	13
p.R241H	p.R408W	1	0.2	1	0	0	-	0	1	0	0	0	0	1	0	12.5
p.R243Q	p.P281L	1	0.2	0	0	1	-	0	0	0	0	1	0	0	-	12
p.R243Q	IVS2+5G>C	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	nd
p.R243Q	IVS10-11G>A	1	0.2	0	0	1	-	1	0	0	0	0	1	0	100	5
p.R243X	p.E390G	1	0.2	0	1	0	-	0	0	0	1	0	0	1	0	36.3
p.R248W	p.R248W	1	0.2	0	1	0	1	0	0	0	1	0	0	1	0	nd
p.R252Q	p.R252Q	1	0.2	0	0	1	1	0	0	0	1	0	0	1	0	3
p.R252Q	p.E390G	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	37
p.R252Q	IVS10-11G>A	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	1.5
p.R252W	p.R261Q	1	0.2	0	1	0	-	0	0	1	0	0	1	0	100	21

Supplementary Table 1 continuing

Allele 1	Allele 2	No of genotypes	GF (%)	mHPA	mPKU	cPKU	Homozygous	Resp @24h	Non-resp @24h	Resp @8h	Non-resp @8h	Not tested	Total resp	Total non-resp	%Resp	PAH act.
p.R261Q	p.T380M	1	0.2	1	0	0	-	0	0	0	0	1	0	0	-	nd
p.R261Q	p.R408W	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	20.3
p.R261Q	p.L333F	1	0.2	0	0	1	-	0	0	0	0	1	0	0	-	35.8
p.R261Q	p.K363Nfs	1	0.2	0	1	0	-	0	0	0	0	1	0	0	-	nd
p.R261Q	p.I306V	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	19.3
p.R261Q	p.A434D	1	0.2	0	1	0	-	0	0	0	1	0	0	1	0	nd
p.R261X	IVS10-11G>A	1	0.2	0	0	1	-	0	1	0	0	0	0	1	0	0
p.R408Q	IVS6-2A>G	1	0.2	0	1	0	-	1	0	0	0	0	1	0	100	28
p.R408W	p.R413P	1	0.2	0	0	1	-	0	0	0	0	1	0	0	-	34
p.R413P	IVS4+1G>T	1	0.2	0	1	0	-	0	0	0	1	0	0	1	0	33
p.R68G	p.A403V	1	0.2	0	0	1	-	0	0	1	0	0	1	0	100	50
p.S16>XfsX1	p.S16>XfsX1	1	0.2	1	0	0	1	0	0	0	0	1	0	0	-	0
p.S16>XfsX1	IVS10-11G>A	1	0.2	0	0	1	-	0	1	0	0	0	0	1	0	0
p.T372S	p.T372S	1	0.2	1	0	0	1	0	0	0	1	0	0	1	0	nd
p.T372S	p.R408W	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	nd
p.T372S	IVS10-11G>A	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	nd
p.T380M	p.R408W	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	nd
p.V177L	IVS11+1G>C	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	nd
p.V230I	p.R408W	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	27
p.V230I	p.E390G	1	0.2	1	0	0	-	1	0	0	0	0	1	0	100	63.8
p.V230I	IVS2+5G>C	1	0.2	1	0	0	-	0	0	0	1	0	0	1	0	27
p.V388M	IVS4+5G>T	1	0.2	0	0	1	-	1	0	0	0	0	1	0	100	nd
p.V388M	IVS2+5G>C	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	nd
p.V388M	IVS10-11G>A	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	nd
p.Y204X	p.Y387H	1	0.2	0	0	1	-	0	1	0	0	0	0	1	0	nd

Supplementary Table 1 continuing

Allele 1	Allele 2	No of genotypes	GF (%)	mHPA	mPKU	cPKU	Homozygous	Resp @24h	Non-resp @24h	Resp @8h	Non-resp @8h	Not tested	Total resp	Total non-resp	%Resp	PAH act.
p.Y204X	p.R241H	1	0.2	0	1	0	-	1	0	0	0	0	1	0	100	13
p.Y204X	IVS10-11G>A	1	0.2	0	0	1	-	0	0	0	1	0	0	1	0	0
p.Y387H	p.Y387H	1	0.2	0	0	1	1	0	0	1	0	0	1	0	100	nd
X6 deletion	X6 deletion	1	0.2	0	0	1	1	0	0	0	1	0	0	1	0	0
Total		**540**		**151**	**125**	**264**	**263**									

GF = genotypes frequency; mHPA = mild HPA; mPKU = mild PKU; cPKU = classic PKU; Resp @24h = Number of responder 24h post challenge; Non-resp @24h = Number of non-responder 24h post challenge; Resp @8h = Number of responder 8h post challenge; Non-resp @8h = Number of non-responder 8h post challenge; nd = not done; For PAH act. see Materials and Methods section

Chapter 2

Genotype-predicted Tetrahydrobiopterin (BH_4)-Responsiveness and Molecular Genetics in Croatian Patients with Phenylalanine Hydroxylase (PAH) Deficiency

Iva Karačić,[1] David Meili,[2] Vladimir Sarnavka,[3] Caroline Heintz,[2] Beat Thöny,[2] Danijela Petković Ramadža,[3] Ksenija Fumić,[4] Duško Mardešić,[1,3] Ivo Barić,[1,3] and Nenad Blau[2 *]

[1]*School of Medicine, Zagreb, Croatia*

[2]*Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Zürich, Switzerland*

[3]*Department of Pediatrics, University Hospital Center, Zagreb, Croatia*

[4]*Clinical Institute of Laboratory Diagnosis, University Hospital Center, Zagreb, Croatia*

Published in Molecular Genetics and Metabolism 2009, 97 (3): 165-71.

Abstract

Specific mutations in the gene encoding phenylalanine hydroxylase (*PAH*), located on chromosome 12q22 - 24.1, are linked to tetrahydrobiopterin (BH_4; sapropterin)-responsive phenylketonuria (PKU). Diagnosis is usually done through the newborn screening for PKU, followed by a BH_4 loading test. So far, more than 60 mutant alleles, presenting with a substantial residual PAH activity (average ~ 47%), were identified in more than 500 patients worldwide. We investigated the predictive value of BH_4-responsive *PAH* mutations in Croatian population. From a group of 127 PKU patients, 62 were selected (based on the genotype) as potentially BH_4-responsive and 39 loaded with BH_4 (20 mg/kg). The overall frequency of BH_4 responsiveness (> 30% blood phenylalanine reduction within 24 h) was 36% (14 out of 39 patients with 23 different genotypes), significantly less than expected. The best responders were patients with mild hyperphenylalaninemia (4/4; 100%), followed by mild PKU (8/9; 89%), and classical PKU (2/26; 8%). The most common BH_4-responsive genotypes were p.E390G/p.R408W and p.P281L/p.E390G. These genotypes correspond for approximately > 30% residual PAH activity. The p.E390G mutation was 100% associated with BH_4 responsiveness, regardless of the second allele (p.R408W, p.P281L, p.F55Lfs, p.L249P). With regard to the predicted relative PAH activity of recombinantly expressed mutant alleles, there was a significant ($p < 0.002$) difference between BH_4-responders and non-responders. In a general Croatian PKU population, disease-causing mutations were identified on 226 alleles (99%). There were 35 different mutations: 21 missense, 8 splice site, 3 nonsense, 2 single nucleotide deletions, and 1 in-frame deletion. Four mutations are reported for the first time: p.E76D, p.L333P, p.G346E, and IVS8-2A>G. Five mutations accounted for over two-thirds of investigated alleles: p.L48S, p.R261Q, p.P281L, p.E390G, and p.R408W. Thus, the Croatian PKU population seems to be more homogenous than some other Mediterranean or Central European populations. This study reveals the importance of a full genotype for the prediction of BH_4 responsiveness. In contrast to previous assumption and with exception of the p.E390G mutation, single allele mutations are not reliable for the selection of potential PKU candidates for pharmacological therapy with BH_4.

Introduction

Phenylketonuria (PKU; OMIM #261600) is an autosomal recessive metabolic disease caused by hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1) deficiency [1]. Over 500 different mutations, identified on PAH gene, are responsible for a large spectrum of clinical phenotypes [2], from mild hyperphenylalaninemia (MHP), a variant that does not require treatment, to classical PKU that leads to severe neurological impairment when untreated. Although phenylalanine restriction has been the mainstay of successful dietary treatment since 1953 when first initiated [3], it imposes a substantial burden on individuals with PKU and the family. This synthetic, highly restrictive diet is associated with a risk of nutritional deficiencies and phenylalanine control, despite good compliance, is sometimes difficult to achieve. However, compliance is often poor, particularly as individuals reach adolescence [4]. Moreover, there is information on poor phenylalanine control before and during pregnancy in women with PKU, which can adversely influence fetal health [5]. Hence there is a need for an alternative treatment of PKU.

BH_4, a catalytic cofactor for PAH, has been shown to activate residual PAH activity and partially restore oxidative Phe metabolism in a substantial number of PKU patients. Although this finding was suggested many years ago [6, 7], not much attention has been paid to this issue until 1999 when Kure et al. [8] reported patients with PAH deficiency who had responded to oral BH_4 intake by lowering their blood Phe levels. Since then, an increasing number of BH_4-responsive PAH-deficient patients has been reported [9 - 22]. Continued treatment with BH_4 in responding patients has been shown to increase Phe tolerance, reduce or eliminate the need for Phe-free protein supplements or even to completely replace the diet [23 - 29]. One of the greatest issues still remains how to identify BH_4-responsive individuals in a large and heterogeneous pool of PAH-deficient patients.

BH_4 loading test result depends on many methodological factors such as preload plasma Phe level, the patient's age at test (newborn vs. older) [12, 18, 30 - 32], Phe intake during the test, amount of administered BH_4 and dosage scheme, cut-off levels of Phe reduction and duration of BH_4 test. An optimized 48-h BH_4 loading protocol, with two BH_4 administrations (20 mg/kg/d) on two consecutive days and with four blood samplings (T_0, T_8, T_{16}, and T_{24}) after BH_4 administration has been proposed for this reason [33]. Nevertheless, one study reported a significant number (> 50%) of initially positive BH_4 responders (Phe reduction > 30% in short-term loading test lasting up to 24 h) who did not respond to long-term BH_4 treatment [27]. Also, there are several reports on patients with no significant response to

single-dose loading test, but with a marked decrease in plasma Phe after several days of BH_4 administration [12, 34]. These inconsistencies stress the need for an additional approach to evaluation of BH_4 responsiveness.

Specific mutations in the *PAH* gene, many of them characterized by substantial residual activity when recombinantly expressed in different cell systems, are repeatedly found to be associated with BH_4 responsiveness [10, 35, 36]. This is in accordance with data from BH_4 loading tests indicating an incidence of BH_4 responsiveness of > 80% in mild variants of PKU patients with an overall incidence of > 40% in general PKU population [37]. Up to 10% of classical PKU patients respond in BH_4 loading test (with a usual 30% cut-off in blood Phe reduction) and they are a more difficult target to properly evaluate BH_4 responsiveness. This is because some severe PKU patients had responded to BH_4 by lowering Phe levels for 20%, which was defined as a significant response for this phenotype. Thus, Fiege and Blau [30] propose to modify the cut-off level for BH_4 responsiveness accordingly to the patient's clinical phenotype. However, there is no accurate correlation between genotype and BH_4 responsiveness, still with many reported responding inconsistencies within the same genotype [36]. So far, mutational analysis provides useful information on potential non-responders comprising two null mutations but the prediction of BH_4 responders remains incomplete [37].

The aim of our study was to provide more information on predictive value of genotype for BH_4 responsiveness and to summarize the mutation spectrum of the *PAH* gene in Croatian PKU population. We initially suggested that the presence of a mutation with *in vitro* substantial residual activity, compared with the wild type enzyme, on at least one *PAH* gene copy would be sufficient for BH_4 responsiveness.

Patients and Methods

Patients

From a group of 127 patients diagnosed with hyperphenylalaninemia (HPA) (the highest blood phenylalanine 300 - 3630 μmol/L) from Croatia in whom *PAH* gene mutation analysis had been done, 39 patients were included in BH_4 loading test. Although we selected 62 patients, only 39 individuals accepted to perform the BH_4 loading test. In four families two sibs were included. Selection criteria were only based on genotype. Inclusion criteria were: (a) presence of at least one BH_4-responsive mutation; or (b) presence of at least one mutation termed as unclear in correlation to BH_4 responsiveness; or (c) presence of at least one mutation with so far unknown response to BH_4. A mutation was classified as BH_4-responsive

if it was present either in homozygous or functional heterozygous form in BH_4 responders from data in different publications (for further explanation on definition of BH_4-responsive or unclear mutations see Zurflüh et al. [37]). Patients with two null mutations (with no residual activity) were excluded from the study. For mutation classification in relation to BH_4 responsiveness and for additional information on *PAH* gene mutations we used data from BIOPKU database (www.bh4.org/BH4DatabasesBiopku.asp) and a locus-specific knowledgebase *PAH*db (www.pahdb.mcgill.ca). There was an almost equal distribution between females (19/39) and males (20/39) (age ranged 1 - 24 years; mean 11 years) entering BH_4 trial. BH_4 deficiency was excluded in all patients by measuring urinary pterins and dried blood dihydropteridine reductase activity. Patients were assigned to one of the three phenotype categories according to the highest plasma Phe concentration before introducing the diet or after protein loading test (180 mg/kg/d of Phe intake over 5 days): 4/39 patients (10%) were classified as MHP (phenylalanine levels ≤ 600 μmol/L), 9/39 patients (23%) were assigned to mild PKU (Phe levels 600 - 1200 μmol/L) and 26/39 patients (67%) to classical PKU (Phe levels > 1200 μmol/L).

BH_4 loading test

BH_4 loading was performed at Department of Pediatrics, University Hospital Center Zagreb, after obtaining an informed consent from all participants or their parents including the approval of the institutional ethics committee. Three or four days before BH_4 loading (classical PKU vs. milder forms) and during the entire testing period patients had no dietary restrictions, moreover, they were encouraged to consume Phe-rich food. BH_4 (6*R*-BH_4 dihydrochloride; Schircks Laboratories, Jona, Switzerland) was administered orally to all patients as a single dose of 20 mg/kg body weight. Blood was collected just before BH_4 administration (T_0), and 8 (T_8), 24 (T_{24}), and 48 h (T_{48}) after the loading. We simplified the criteria suggested by Fiege et al. [17] to define BH_4-responders as follows: ‘‘responder”, reduction of blood Phe by ≥ 30% within 24 h and ‘‘slow responder”, reduction of blood Phe by < 20% at T_8, and ≥ 20% but < 30% at T_{24}. One patient was classified as ‘‘not clear” with the reduction of blood Phe by ≥ 30% at T_8, and < 20% at T_{24}. No side effects were observed during the BH_4 loading test. Phe and BH_4 were measured from dried blood spots; Phe was analyzed using tandem-mass spectrometry and BH_4 was measured according to the method previously published [38].

Mutational analysis

One hundred and fourteen families with HPA (127 patients), all but four patients detected by Guthrie test within neonatal screening program, were enrolled in a comprehensive analysis of *PAH* gene mutations in Croatia in the last 17 years. According to the previously mentioned criteria, 78% of patients suffer from classical PKU, 14% from mild PKU whilst MHP phenotype is present in only 8%. Analyzed patients comprise 78% of total Croatian PKU population. Patients and/or parents signed informed consent for mutational analysis. Genotyping was performed as follows: DNA was isolated from dried blood spots using the QIAamp DNA Micro Kit (Qiagen). PCR was performed using Hot FirePol DNA Polymerase (Solis Biodyne) and standard thermal cycling, i.e., 15 min denaturation at 95°C followed by 37 cycles of 30 s at 95°C, 45 s at 56°C, 45 s at 72°C, and final incubation for 10 min at 72 °C (on a Gene-Amp PCR System 9700 (Applied Biosystems)). Primers flanking all exons are listed in Table 2.1. The same primers were used to directly sequence the amplified products with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were purified by gel filtration (using MultiScreen HV 96-well filter plates from Millipore with Sephadex G-50 (GE Healthcare)) and analyzed on a 3130xl Genetic Analyzer (Applied Biosystems). Sequencing results were compared with the wild type sequence of the *PAH* gene (Accession numbers NM_000277.1 and NG_008690.1) using the Mutation Surveyor (Demo) Software v3.20 (SoftGenetics). Gene analysis of 39 PKU families (included in this study) was done elsewhere as described in two previous publications on PKU mutations in Croatia [39, 40].

The relative PAH activity of mutant alleles was calculated from the data published in the BIOPKUdb (www.biopku.org). It was calculated as a sum of activities for alleles 1 and 2, when expressed recombinantly in different cell systems, divided by two. For alleles expressed in more than one cell system, an average PAH activity was used.

Statistical analysis

Statistical analysis was performed using WinSTAT 2007.1 for Excel (R. Fitch Software, Germany). Wilcoxon test was used to compare the predicted relative PAH activity between BH_4-responders and non-responders.

Table 2.1: Primers used for PCR-amplification and sequencing of all 13 exons plus flanking intronic regions of the human *PAH* gene (see also 'Patients and methods').

Exon[a]	Sequence (5′ > 3′)	Reference
1F	CGTGCTGTTTGCAAACCTGC	This work
1R	TGGAGGCCCAAATTCCCCTAACTG	[44]
2F	TGATCATTTAATTGCCCTGGA	This work
2R	GCCTGTTCCAGATCCTGTGT	This work
3F	GCCTGCGTTAGTTCCTGTGA	[44][b]
3R	CTTATGTTGCAAAATTCCTC	[44]
4F	GCCATGTTCTGCCAATCTGT	This work
4R	ATCTCATCCTACGGGCCAT	Denmark[c]
5F	TCATGGCTTTAGAGCCCCCA	[44]
5R	AGGCTAGGGGTGTGTTTTTC	[44][b]
6F	CCGACTCCCTCTGCTAACCT	[44][b]
6R	CAATCCTCCCCCAACTTTCT	[44]
7F	TAGCGTCAAAGCCTATGTCC	This work
7R	AAACCTCATTCTTGCAGCAG	This work
8F	TGGCTTAAACCTCCTCCCCT	[44][b]
8R	CTGGGCTCAACTCATTTGAG	[44]
9F	ATGGCCAAGTACTAGGTTGG	[44]
9R	GAGGGCCATAGACTATAGCA	[44][b]
10F	ACACACCCCAAAATAATGCT	This work
10R	GAGTTCCCAGGTTGCATATC	This work
11F	TGAGAGAAGGGGCACAAATG	[44]
11R	CCACCCACAGATGAGTGGCA	This work
12F	TTCTCCAAATGGTGCCCTTC	Denmark[c]
12R	ACTGAGAAACCGAGTGGCCT	Denmark[c]
13F	GACACTTGAAGAGTTTTTGC	[44][b]
13R	TTTTCGGACTTTTTCTGAT	This work

[a] F, forward; R, reverse.
[b] Without GC clamp.
[c] Personal communication Pia Hougaard and Lisbeth Birk Møller, Kennedy Institute, Denmark.

Results

Mutational spectrum in Croatian PKU population

In our mutation frequency assessment we included 114 PKU families. In one family there were three independent mutant alleles (affected child and uncle) what was the reason for investigating the total of 229 independent alleles. Disease-causing mutation was identified on 226 alleles, corresponding to a diagnostic efficiency of approximately 99%. There were 35 different mutations, including 21 missense mutations, 8 splice site, 3 nonsense, 2 single nucleotide deletions, and one in-frame deletion. The commonest PKU mutation p.R408W accounted for 36% of mutant alleles. Following mutations p.L48S, p.P281L, p.E390G, p.R261Q, and p.R158Q accounted for 10%, 8%, 7%, 6%, and 5.5% of mutant alleles, respectively. Five mutations accounted for over two-thirds of investigated alleles (Table 2.2). Four mutations have not been previously reported: p.E76D, p.G346E, p.L333P, and IVS8-2A>G.

Table 2.2: The most frequent mutations in Croatian PKU population, which account for over two-thirds of the investigated population (229 independent chromosomes)

Mutation	Number of alleles	Frequency (number of independent chromosomes) (%)
p.R408W (c.1222C > T)	82	36
p.L48S (c.143T > C)	23	10
p.P281L (c.842C > T)	18	8
p.E390G (c.1169A > G)	16	7
p.R261Q (c.782G > A)	14	6
Total	153	67

Croatian PKU population is more homogenous than some Mediterranean or Central European populations as Zschocke et al. [40] already described. We found homozygosity in 24% of genotypes (for following mutations: p.R408W, p.P281L, p.E390G, p.L249P, p.L48S, and p.R158Q) and only 10 allelic combinations accounted for over 50% of investigated families in Croatia (Table 2.3). Thus, the investigation of these allelic combinations in accordance to BH_4 response was of great interest for our population.

Table 2.3: The most frequent allelic combinations in Croatia that account for over half of the investigated PKU population (114 families)

Genotype	Number of patients	Frequency in investigated population (%)
p.R408W/p.R408W (c.1222C > T/c.1222C > T)	15	13
p.R408W/p.L48S (c.1222C > T/c.143T > C)	8	7
p.R408W/p.E390G (c.1222C > T/c.1169A > G)	6	5
p.R408W/p.P281L (c.1222C > T/c.842C > T)	5	4.5
p.R408W/p.R261Q (c.1222C > T/c.782G > A)	5	4.5
p.L48S/p.R261Q (c.143T > C/c.782G > A)	4	3.5
p.L48S/p.R158Q (c.143T > C/c.473G > A)	4	3.5
p.R408W/p.R158Q (c.1222C > T/c.473G > A)	4	3.5
p.R408W/IVS12 + 1G > A (c.1222C > T/ c.1315 + 1G > A)	4	3.5
p.P281L/p.E390G (c.842C > T/c.1169A > G)	3	2.5
Total	58	50.5

Responsiveness to BH_4

The outcome of the loading test with BH_4 in 39 PKU patients with 23 different genotypes is summarized in Table 2.4 (according to biochemical and genetic phenotype and its‘ mean predicted residual PAH activity [41]). The prevalence of BH_4 responsiveness (at least 30% cut-off of phenylalanine reduction within 24 h) was only 36% (14 of 39 patients). Additionally, there were two slow responders who decreased Phe for 26.4% and 24% within 24 h post loading (patient 1 and 25). The prevalence of BH_4-responders in different phenotype groups was as follows: 100% (4/4 patients) in MHP group, 89% (8/9 patients) in mild PKU group, and 8% (2/26 patients) in classical PKU group. All MHP patients and 5/8 responding mild PKU patients (patients 2, 3, 4, 5, and 39) showed significant Phe reduction (> 50%) already 8 h post loading. In severe phenotypes that responded (two patients plus one slow responder) phenylalanine reduction was significantly (over 20%) expressed 24 h post loading. Interestingly, patient 6 with the same genotype (p.E390G/p.R408W) (and disease severity, accordingly) as patients 2, 3, 4, and 5 showed significantly slower and less effective response to BH_4. We could not explain this by differences in test performance or BH_4 pharmacokinetics, and to our knowledge such poor response of this genotype has never been described before. It is, however, possible that in this patient Phe intake during the test was lower than in other patients with the same genotype. In all patients blood BH_4 levels increased from initial 1.25 ± 1.11 nmol/g Hb (mean ± SD) to 6.06 ± 4.79 nmol/g Hb 8 h after BH_4 administration and decreased to 2.07 ± 1.16 nmol/g Hb after 24 h and 1.64 ± 1.47 nmol/g Hb after 48 h.

Thirty-five patients from this study (see Table 2.4) had at least one mutation with known *in vitro* residual activity of 10% or more (compared with the wild type enzyme), and we assumed that each patient from this group would respond to BH_4. However, only 14/35 (40%) of these patients responded and one patient was labeled “not clear” (patient 19) as he responded just at T_8, and then elevated Phe significantly at T_{24}. This was not estimated as reliable response to BH_4. Additionally, we loaded with BH_4 four patients with p.L249P, mutation with unknown response to BH_4 (and with no *in vitro* studies on residual enzyme activity). To our knowledge, this mutation was only described in Croatian PKU population. p.L249P was first described by Zschocke et al. [40]. It accounts for 3.5% of mutant alleles and, according to the results of BH_4 loading test, it does not have any substantial residual enzyme activity. Namely, this mutation was associated with BH_4 responsiveness only when in allelic combination with p.E390G (so far 100% responsive mutation), but did not show any response in homozygous or functional heterozygous condition.

Table 2.4: Tetrahydrobiopterin loading test in 39 Croatian PKU patients selected according to genotype data and predictive value of genotype on BH_4 responsiveness according to predicted relative residual PAH activity

Patient number	Maximal Phe levels[a] (phenotype)	Allele 1	Allele 2	Phe 0 h	Phe 8 h	Phe 24 h	Phe 48 h[b]	Phe reduction 8 h (%)	Phe reduction 24 h (%)	PAH activity[c]	BH_4 responsiveness
24	1355 (cPKU)	**p.L48S** c.143T > C	**p.L48S** c.143T > C	315	237	140	292	24,8	55,6	39.0	Responder
25	1520 (cPKU)	**p.L48S** c.143T > C	**p.L48S** c.143T > C	655	740	498	582	−13,0	24,0	39.0	Slow-responder
29	1023 (mPKU)	**p.L48S** c.143T > C	**p.R261Q** c.782G > A	1211	nd	759	1060	nd	37,3	39.0	Responder
2	617 (mPKU)	**p.E390G** c.1169A > G	p.R408W c.1222C > T	437	179	239	341	59,0	45,3	37.5	Responder
3	677 (mPKU)	**p.E390G** c.1169A > G	p.R408W c.1222C > T	608	181	115	329	70,2	81,1	37.5	Responder
4[1]	1180 (mPKU)	**p.E390G** c.1169A > G	p.R408W c.1222C > T	411	132	94	340	67.9	77.1	37.5	Responder
5[1]	607 (mPKU)	**p.E390G** c.1169A > G	p.R408W c.1222C > T	519	216	118	261	58,4	77,3	37.5	Responder
6	871 (mPKU)	**p.E390G** c.1169A > G	p.R408W c.1222C > T	389	310	247	363	20,3	36,5	37.5	Responder
7	750 (mPKU)	**p.E390G** c.1169A > G	p.P281L c.842C > T	265	225	146	304	15,1	44,9	37.0	Responder
8	600 (MHP)	**p.E390G** c.1169A > G	p.P281L c.842C > T	332	148	133	412	55,4	59,9	37.0	Responder
9	600 (MHP)	**p.E390G** c.1169A > G	p.F55Lfs c.165delT	285	124	90	239	56,5	68,4	36.5	Responder
10	360 (MHP)	**p.E390G** c.1169A > G	p.L249P c.746T > C	345	138	136	382	60,0	60,6	(36.5)	Responder
27	>1210 (cPKU)	**p.L48S** c.143T > C	**p.R158Q** c.473G > A	1298	1024	908	764	21,1	30,0	24.5	Responder
28	2718 (cPKU)	**p.L48S** c.143T > C	**p.R158Q** c.473G > A	956	951	1069	1016	0,5	−11,8	24.5	Non-responder
17[3]	1658 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	762	733	876	895	3,8	−15,0	20.5	Non-responder
18[3]	2050 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	1281	1298	1218	1370	−1,3	4,9	20.5	Non-responder
19	>1210 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	852	521	763	948	38,8	10,4	20.5	Not clear
20	2153 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	1157	1241	1365	1367	−7,3	−18,0	20.5	Non-responder
21	2118 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	900	859	811	940	4,6	9,9	20.5	Non-responder
22	2039 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	1150	1392	1472	1414	−21,0	−28,0	20.5	Non-responder
23	1920 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	898	942	855	1308	−4,9	4,8	20.5	Non-responder
30	2492 (cPKU)	p.R408W c.1222C > T	**p.R261Q** c.782G > A	1096	1361	1160	1256	−24,2	−5,8	20.5	Non-responder
31	3260 (cPKU)	p.R408W c.1222C > T	**p.R261Q** c.782G > A	1084	1080	1150	1088	0,4	− 6,1	20.5	Non-responder
16	>1210 (cPKU)	**p.L48S** c.143T > C	p.P281L c.842C > T	1092	1373	1336	1169	−25,7	−22,3	20.0	Non-responder
35	>1210 (cPKU)	p.R408W c.1222C > T	**p.V245A** + **p.R241C** c.734T > C + c.721C > T	1203	1304	1225	1145	−8,4	−1,8	20.0	Non-responder
26	1466 (cPKU)	**p.L48S** c.143T > C	IVS4 + 5G > T c.441 + 5G > T	879	938	863	944	−6,7	1,8	19.5	Non-responder
32[4]	1573 (cPKU)	p.K363 > Nfs c.1089delG	**p.R261Q** c.782G > A	1152	937	1068	1203	18,7	7,3	19.5	Non-responder
33[4]	1452 (cPKU)	p.K363 > Nfs c.1089delG	**p.R261Q** c.782G > A	1097	954	1006	1025	13,0	8,3	19.5	Non-responder
38	420 (MHP)	**p.I306V** c. 916A > G	p.R408W c.1222C > T	255	83	64	214	67,5	74,9	20.5	Responder
39	720 (mPKU)	**p.I306V** c. 916A > G	p.R111X c.331C > T	335	118	187	283	64,8	44,2	19.5	Responder
34	1211 (cPKU)	p.R408W c.1222C > T	**p.F39del** c.115_117delTTC	1358	1451	1281	1354	−6,8	5,7	11.0	Non-responder
36	2698 (cPKU)	p.R408W c.1222C > T	**p.R158Q** c.473G > A	1377	1164	1158	1118	15,5	15,9	6.0	Non-responder
14	>1210 (cPKU)	p.L249P c.746T > C	**p.R158Q** c.473G > A	632	674	550	741	−6,6	13,0	(5.0)	Non-responder
37	>1210 (cPKU)	**p.R158Q** c.473G > A	?	738	738	784	780	0,0	−6,2	(5.0)	Non-responder
13	1984 (cPKU)	p.L249P c.746T > C	p.R408W c.1222C > T	871	880	792	874	−1,0	9,1	(1.0)	Non-responder
15	2940 (cPKU)	p.L249P c.746T > C	p.P281L c.842C > T	1033	962	1114	1124	6,9	−7,8	(0.5)	Non-responder
1	1082 (mPKU)	**p.I306V** c. 916A > G	p.R261X c.781C > T	295	255	217	263	13,6	26,4	19.5	Slow-responder
11[2]	1306 (cPKU)	p.L249P c.746T > C	p.L249P c.746T > C	1150	1215	1239	1252	−5,7	−7,7	?	Non-responder
12[2]	3055 (cPKU)	p.L249P c.746T > C	p.L249P c.746T > C	1185	1241	1225	1332	−4,7	−3,4	?	Non-responder

nd: not done; ?: not known; 1, 2, 3, 4: pairs of siblings

[a] The highest Phe pretreatment concentration (μmol/L) or the highest Phe in the protein Phe test (180 mg/kg/d) if performed; mild hyperphenylalaninemia (MHP), mild phenylketonuria (mPKU), and classical PKU (cPKU).

[b] 48-h Phe value as test control - if Phe increased 48 h after BH_4 administration as expected regarding BH_4 pharmacokinetics, loading test was considered more reliable.

[c] Predicted relative residual PAH activity (sum of *in vitro* expressed residual activities of alleles 1+2 divided by 2). Data from the BIOPKU database (www.biopku.org). Values in brackets show data calculated from only one allele (second allele not known). Mutations in bold are defined as BH_4-responsive [37]

Analysis of predicted relative PAH activities revealed a significant difference ($p < 0.002$) between BH_4-responders and non-responders (Figure 2.1). The median relative PAH activity was 37.2% in BH_4-responder (5th - 95th percentile: 20.0 - 39.0%) and 20.0% in BH_4 non-responder group (5th - 95th percentile: 0.5 - 24.3%).

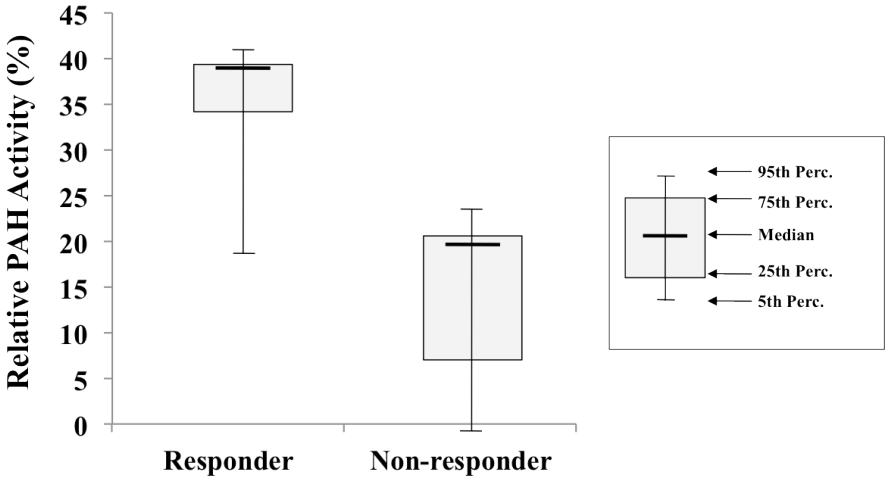


Figure 2.1: Comparison of the predicted relative PAH activity in patients with the BH_4-responsive PKU (n = 14) and in non-responders (n = 20). For definition of the predicted relative PAH activity, see ‘‘Patients and methods” and Table 4. Three patients (two slow-responder and one not clear) were not included in the comparison. In four non-responder patients the relative PAH activity was calculated from only one allele, as well as for one responder. In two patients no data were available for the relative PAH activity.

Discussion

This is the first study on BH_4 responsiveness in a cohort of HPA patients where selection criteria for BH_4 loading test were only based on genotype information. We predicted BH_4 responsiveness in individuals with at least one mutation expressing in vitro substantial residual activity (> 10%). This prediction was based on a current knowledge that specific mutation with some residual activity would be considered as the major determinant of BH_4-responding phenotype. Mutation-predicted prevalence of BH_4 responsiveness was, however, almost threefold higher than prevalence data obtained after our BH_4 loading test. This finding reveals the importance of the complete *PAH* genotype instead of just a single responsive mutation in predicting the BH_4 responsiveness.

Croatia has relatively homogenous PKU population with around 75% of families comprising classical PKU. Mutational background confirms this finding as the most common mutation p.R408W accounts for 36% of mutant alleles, with p.R408W homozygosity rate of 13%. Total homozygosity rate (24%) is higher than in majority of other European

populations (according to data summarized in Guldberg et al. [42]). However, we believed there could be a significant number of BH_4-responsive patients in this population, not just among 22% of milder forms, but also in severe phenotypes. This prediction was based on the fact that ‘‘milder” mutations such as p.L48S, p.E390G, p.R261Q, and p.R158Q were following p.R408W with allelic frequency of 10%, 7%, 6%, and 5.5%, respectively, in Croatian PKU population.

Our results reveal, however, negative impact of the most frequent mutation, p.R408W, on the second allele with BH_4-responsive mutation (p.L48S, p.R261Q, or p.R158Q). These three mutations have been repeatedly reported as inconsistent in BH_4 responsiveness [10, 13, 16, 18, 36], and they cannot be regarded as dominant in compound heterozygous patients as their behavior highly depends on the other mutation. Moreover, we observed to some extent consistent pattern in BH_4 responsiveness for specific combinations of “inconsistent” mutations p.L48S and p.R261Q. They are non-responders (except patient 19 regarded as “not clear”) in functional hemizygous form, but found as BH_4-responders in homozygous form (including one slow responder), in compound heterozygosity with MHP mutation as well as in *in trans* combination of this two mutations (p.L48S/p.R261Q). Although similar results were reported in many publications, there are several exceptions such as a report of BH_4-responsive p.R261Q/p.R243X genotype by Spaapen and Estela Rubio-Gozalbo [43] as well as reported non-responder with p.L48S homozygosity by Fiori et al. [16]. Both p.L48S homozygotes (patient 24 and 25) belong to milder form of classical PKU as they increase Phe much slower than typical classical PKU patients when not on diet, but however they can eventually reach high Phe levels (see their pretreatment Phe concentrations from Table 2.4). As reported by Leuzzi et al. [18] and confirmed in our study, p.L48S homozygotes sometimes show slow response to BH_4 (at T_{24} Phe reduction of 24%). Thus, in a case of this genotype a 30% cut-off in Phe reduction would not be always reliable to estimate BH_4 responsiveness. Trefz et al. [36] found p.R158Q mutation as the most inconsistent of all mutations (500 alleles investigated) in responding to BH_4. Our results confirm this observation, especially in case of compound heterozygosity with p.L48S (patient 27 and 28). Functional hemizygous patients for p.R158Q result in BH_4 non-responsiveness. Our study confirms the definition of p.E390G mutation as 100% responsive allele. To our knowledge, this mutation has never so far been described as BH_4 non-responsive, regardless of the second allele. The similar was observed for much less frequent p.I306V mutation (full responsiveness in two patients and “slow” responsiveness in the third loaded patient), although the response of the latter needs to be evaluated on more patients.

Our findings reveal following features: one mutation is sufficient to estimate response to BH_4 just in case of MHP mutations (e.g., p.E390G). In all other cases mutational combination (i.e., complete genotype) should be used to indicate and adjust, still not standardized, BH_4 loading test. On the basis of allelic mutational combination we were able to predict BH_4-response for the majority of patients. The exceptions were genotypes with mutations with unknown response to BH_4 and so far unmeasured *in vitro* residual activity (p.L249P), with in *cis* mutations (p.V245A+p.R241C), and highly inconsistent genotype (p.L48S/p.R158Q). Genotype information is also useful for the selection of a target population among patients with classical PKU (i.e., by excluding patients with two null mutations), as well as to point to patients who are probable slow-responders not to be missed by too rigorous loading protocol (e.g., some p.L48S homozygotes).

The predicted relative PAH activities (Table 2.4 and Figure 2.1) were significantly higher in BH_4-responders, compared with the non-responder group. It seems that a substantial residual PAH activity resulting from a combination of both alleles is needed to predict 100% responsiveness in PKU patients.

Acknowledgments

This work was supported by Grant No. 108-1081870-1885 of the Ministry of Science, Education and Sport of Republic of Croatia, by the Swiss National Science Foundation Grant No. 3100A0-1199852/1, and in part by a grant from BioMarin Pharmaceuticals Inc., Novato, CA (to N.B.). We thank our nurses from The Division of Genetics and Metabolism, Department of Pediatrics in University Hospital Center Zagreb for technical assistance, Thomas Polanski for measurement of pterins, and Rita Denz for measurement of amino acids.

References

[1] C.R. Scriver, S. Kaufman, Hyperphenylalaninemia: phenylalanine hydroxylase deficiency, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, B. Vogelstein (Eds.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 1667–1724.

[2] C.R. Scriver, The PAH gene, phenylketonuria, and a paradigm shift, Hum. Mutat. 28 (2007) 831–845.

[3] H. Bickel, J.W. Gerrard, E.M. Hickmans, Influence of phenylalanine intake on phenylketonuria, Lancet (1953) 812–819.

[4] J.H. Walter, F.J. White, S.K. Hall, A. MacDonald, G. Rylance, A. Boneh, D.E. Francis, G.J. Shortland, M. Schmidt, A. Vail, How practical are recommendations for dietary control in phenylketonuria?, Lancet 360 (2002) 55–57.

[5] A.S. Brown, P.M. Fernhoff, S.E. Waisbren, D.M. Frazier, R. Singh, F. Rohr, J.M. Morris, A. Kenneson, P. MacDonald, M. Gwinn, M. Honein, S.A. Rasmussen, Barriers to successful dietary control among pregnant women with phenylketonuria, Genet. Med.4 (2002) 84–89.

[6] S. Milstien, S. Kaufman, Studies on the phenylalanine hydroxylase system in liver slices, J. Biol. Chem. 250 (1975) 4777–4781.

[7] A. Niederwieser, H.C. Curtius, Tetrahydrobiopterin deficiencies in hyperphenylalaninemia, in: H. Bickel, U. Wachtel (Eds.), Inherited Diseases of Amino Acid Metabolism, Georg Thieme, Stuttgart, 1985, pp. 104–121.

[8] S. Kure, D.C. Hou, T. Ohura, H. Iwamoto, S. Suzuki, N. Sugiyama, O. Sakamoto, K. Fujii, Y. Matsubara, K. Narisawa, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, J. Pediatr. 135 (1999) 375–378.

[9] C. Bernegger, N. Blau, High frequency of tetrahydrobiopterin-responsiveness among hyperphenylalaninemias: a study of 1919 patients observed from 1988 to 2002, Mol. Genet. Metab. 77 (2002) 304–313.

[10] A.C. Muntau, W. Röschinger, M. Habich, H. Demmelmair, B. Hoffmann, C.P. Sommerhoff, A.A. Roscher, Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria, N. Engl. J. Med. 347 (2002) 2122–2132.

[11] M. Lindner, R. Steinfeld, P. Burgard, A. Schulze, E. Mayatepek, J. Zschocke, Tetrahydrobiopterin sensitivity in German patients with mild phenylalanine hydroxylase deficiency, Hum. Mutat. 21 (2003) 400.

[12] H. Shintaku, S. Kure, T. Ohura, Y. Okano, M. Ohwada, N. Sugiyama, N. Sakura, I. Yoshida, M. Yoshino, Y. Matsubara, K. Suzuki, K. Aoki, T. Kitagawa, Long-term treatment and diagnosis of tetrahydrobiopterin-responsive hyperphenylalaninemia with a mutant phenylalanine hydroxylase gene, Pediatr. Res. 55 (2004) 425–430.

[13] B. Perez-Duenas, M.A. Vilaseca, A. Mas, N. Lambruschini, R. Artuch, L. Gomez, J. Pineda, A. Gutierrez, M. Mila, J. Campistol, Tetrahydrobiopterin responsiveness in patients with phenylketonuria, Clin. Biochem. 37 (2004) 1083–1090.

[14] L.R. Desviat, B. Pérez, A. Bèlanger-Quintana, M. Castro, C. Aguado, A. Sánchez, M.J. García, M. Martínez-Pardo, M. Ugarte, Tetrahydrobiopterin responsiveness: results of the BH_4 loading test in 31 Spanish PKU patients and correlation with their genotype, Mol. Genet. Metab. 82 (2004) 157–162.

[15] J.J. Mitchell, B. Wilcken, I. Alexander, C. Ellaway, H. O'Grady, V. Wiley, J. Earl, J. Christodoulou, Tetrahydrobiopterin-responsive phenylketonuria: the New South Wales experience, Mol. Genet. Metab. 86 (2005) 81–85.

[16] L. Fiori, B. Fiege, E. Riva, M. Giovannini, Incidence of BH_4-responsiveness in phenylalanine-hydroxylase-deficient Italian patients, Mol. Genet. Metab. 86 (2005) 67–74.

[17] B. Fiege, L. Bonafé, D. Ballhausen, M. Baumgartner, B. Thöny, D. Meili, L. Fiori, M. Giovannini, N. Blau, Extended tetrahydrobiopterin loading test in the diagnosis of cofactor-responsive phenylketonuria: a pilot study, Mol. Genet. Metab. 86 (2005) 91–95.

[18] V. Leuzzi, C. Carducci, C. Carducci, F. Chiarotti, C. Artiola, T. Giovanniello, I. Antonozzi, The spectrum of phenylalanine variations under tetrahydrobiopterin load in subjects affected by phenylalanine hydroxylase deficiency, J. Inherit. Metab. Dis. 29 (2006) 38–46.

[19] S. Yildirim, A. Tokatli, E. Yilmaz, T. Coskun, Assessment of tetrahydrobiopterin responsiveness in Turkish hyperphenylalaninemic patients, Turk. J. Pediatr. 49 (2007) 1–6.

[20] L. Wang, S. Surendran, K. Michals-Matalon, G. Bhatia, S. Tanskley, R. Koch, J. Grady, S.K. Tyring, R.C. Stevens, F. Guttler, R. Matalon, Mutations in the regulatory domain of phenylalanine hydroxylase and response to tetrahydrobiopterin, Genet. Test. 11 (2007) 174–178.

[21] M.D. Boveda, M.L. Couce, D.E. Castineiras, J.A. Cocho, B. Perez, M. Ugarte, J.M. Fraga, The tetrahydrobiopterin loading test in 36 patients with hyperphenylalaninaemia: evaluation of response and subsequent treatment, J. Inherit. Metab. Dis. 30 (2007) 812.

[22] B.K. Burton, D.K. Grange, A. Milanowski, G. Vockley, F. Feillet, E.A. Crombez, V. Abadie, C.O. Harding, S. Cederbaum, D. Dobbelaere, A. Smith, A. Dorenbaum, The response of patients with phenylketonuria and elevated serum phenylalanine to treatment with oral sapropterin dihydrochloride (6Rtetrahydrobiopterin): a phase II, multicentre, open-label, screening study, J. Inherit. Metab. Dis. 30 (2007) 700–707.

[23] R. Cerone, M.C. Schiaffino, A.R. Fantasia, M. Perfumo, L. Birk Moller, N. Blau, Long-term follow-up of a patient with mild tetrahydrobiopterin-responsive phenylketonuria, Mol. Genet. Metab. 81 (2004) 137–139.

[24] J.B. Hennermann, C. Bührer, N. Blau, B. Vetter, E. Mönch, Long-term treatment with tetrahydrobiopterin increases phenylalanine tolerance in children with severe phenotype of phenylketonuria, Mol. Genet. Metab. 86 (2005) 86–90.

[25] N. Lambruschini, B. Perez-Duenas, M.A. Vilaseca, A. Mas, R. Artuch, R. Gassio, L. Gomez, A. Gutierrez, J. Campistol, Clinical and nutritional evaluation of phenylketonuric patients on tetrahydrobiopterin monotherapy, Mol. Genet. Metab. 86 (2005) 54–60.

[26] F.K. Trefz, D. Scheible, G. Frauendienst-Egger, H. Korall, N. Blau, Long-term treatment of patients with mild and classical phenylketonuria by tetrahydrobiopterin, Mol. Genet. Metab. 86 (2005) 75–80.

[27] H. Levy, A. Milanowski, A. Chakrapani, M. Cleary, P. Lee, F.K. Trefz, C.B. Whitley, F. Feillet, A.S. Feigenbaum, J.D. Bebchuk, H. Christ-Schmidt, A. Dorenbaum, Efficacy of sapropterin dihydrochloride (tetrahydrobiopterin, 6R-BH_4) for reduction of phenylalanine concentration in patients with phenylketonuria: a phase III randomized placebo-controlled study, Lancet 370 (2007) 504–510.

[28] P. Lee, E.P. Treacy, E. Crombez, M. Wasserstein, L. Waber, J. Wolff, U. Wendel, A. Dorenbaum, J. Bebchuk, H. Christ-Schmidt, M. Seashore, M. Giovannini, B.K. Burton, A.A. Morris, Safety and efficacy of 22 weeks of treatment with sapropterin dihydrochloride in patients with phenylketonuria, Am. J. Med. Genet.A 146A (2008) 2851–2859.

[29] A. Burlina, N. Blau, Effect of BH_4 supplementation on phenylalanine tolerance, J. Inherit. Metab. Dis. 32 (2009) 40–54.

[30] B. Fiege, N. Blau, Assessment of tetrahydrobiopterin (BH_4)-responsiveness in phenylketonuria, J. Pediatr. 150 (2007) 627–630.

[31] H. Levy, B. Burton, S. Cederbaum, C. Scriver, Recommendations for evaluation of responsiveness to tetrahydrobiopterin (BH(4)) in phenylketonuria and its use in treatment, Mol. Genet. Metab. 92 (2007) 287–291.

[32] N. Blau, Defining tetrahydrobiopterin (BH_4)-responsiveness in PKU, J. Inherit. Metab. Dis. 31 (2008) 2–3.

[33] N. Blau, A. Belanger-Quintana, M. Demirkol, F. Feillet, M. Giovannini, A. MacDonald, F.K. Trefz, F.J. van Spronsen, Optimizing the use of sapropterin (BH_4) n the management of phenylketonuria, Mol. Genet. Metab. 96 (2009) 158–163.

[34] U. Lässker, J. Zschocke, N. Blau, R. Santer, Tetrahydrobiopterin responsiveness in phenylketonuria. Two new cases and a review of molecular genetic findings, J. Inherit. Metab. Dis. 25 (2002) 65–70.

[35] N. Blau, H. Erlandsen, The metabolic and molecular bases of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, Mol. Genet. Metab. 82 (2004) 101–111.

[36] F.K. Trefz, D. Scheible, H. Gotz, G. Frauendienst-Egger, Significance of genotype in tetrahydrobiopterin-responsive phenylketonuria, J. Inherit. Metab. Dis. 32 (2009) 22–26.

[37] M.R. Zurflüh, J. Zschocke, M. Lindner, F. Feillet, C. Chery, A. Burlina, R. Stevens, B. Thöny, N. Blau, Molecular genetics of tetrahydrobiopterin responsive phenylalanine hydroxylase deficiency, Hum. Mutat. (2008) 167–175.

[38] M. Zurflüh, M. Giovannini, L. Fiori, B. Fiege, Y. Gokdemir, T. Baykal, L. Kierat, K.H. Gärtner, B. Thöny, N. Blau, Screening for tetrahydrobiopterin deficiencies using dried blood spots on filter paper, Mol. Genet. Metab. 86 (2005) 96–103.

[39] I. Baric, D. Mardesic, G. Gjuric, V. Sarnavka, B. Gobel-Schreiner, U. Lichter-Konecki, D.S. Konecki, F.K. Trefz, Haplotype distribution and mutations at the PAH locus in Croatia, Hum. Genet. 90 (1992) 155–157.

[40] J. Zschocke, A. Preusse, V. Sarnavka, K. Fumic, D. Mardesic, G.F. Hoffmann, I. Baric, The molecular basis of phenylalanine hydroxylase deficiency in Croatia, Hum. Mutat. 21 (2003) 399.

[41] M. Bik-Multanowski, J.J. Pietrzyk, B. Didycz, A. Szymczakiewicz-Multanowska, Development of a model for assessment of phenylalanine hydroxylase activity in newborns with phenylketonuria receiving tetrahydrobiopterin: a potential for practical implementation, Mol. Genet. Metab. 94 (2008) 389–390.

[42] P. Guldberg, F. Rey, J. Zschocke, V. Romano, B. Francois, L. Michiels, K. Ullrich, Hoffmann, P. Burgard, H. Schmidt, C. Meli, E. Riva, I. Dianzani, A. Ponzone, Rey, F. Güttler, A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype, Am. J. Hum. Genet. 63 (1998) 71–79.

[43] L.J. Spaapen, M. Estela Rubio-Gozalbo, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, state of the art, Mol. Genet. Metab. 78 (2003) 93–99.

[44] P. Guldberg, V. Romano, N. Ceratto, P. Bosco, M. Ciuna, A. Indelicato, F. Mollica, C. Meli, M. Giovannini, E. Riva, et al., Mutational spectrum of phenylalanine hydroxylase deficiency in Sicily: implications for diagnosis of hyperphenylalaninemia in southern Europe, Hum. Mol. Genet 2 (1993) 1703–1707.

Chapter 3

Quantification of Phenylalanine Hydroxylase Activity by Isotope-Dilution Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry

Caroline Heintz[a], Heinz Troxler[a], Aurora Martinez[b], Beat Thöny[a,c,d], Nenad Blau[a,c,d,e,*]

[a] *Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Zürich, Switzerland*

[b] *Department of Biomedicine, University of Bergen, Bergen, Norway*

[c] *Zürich Center for Integrative Human Physiology (ZIHP), Zürich, Switzerland*

[d] *Research Center for Children (RCC), Zürich, Switzerland*

[e] *Division of Metabolism, University Children's Hospital, Heidelberg, Germany*

Published in Molecular Genetics and Metabolism 2012, 105(4): 559-65.

Abstract

Background: Residual phenylalanine hydroxylase (PAH) activity is the key determinant for the phenotype severity in phenylketonuria (PKU) patients and correlates with the patient's genotype. Activity of in vitro expressed mutant PAH may predict the patient's phenotype and response to tetrahydrobiopterin (BH_4), the cofactor of PAH.

Methods: A robust LC-ESI-MSMS PAH assay for the quantification of phenylalanine and tyrosine was developed. We measured PAH activity a) of the PAH mutations p.Y417C, p.I65T, p.R261Q, p.E280A, p.R158Q, p.R408W, and p.E390G expressed in eukaryotic COS-1 cells; b) in different cell lines (e.g. Huh-7, Hep3B); and c) in liver, brain, and kidney tissue from wild type and PKU mice.

Results: The PAH assay was linear for phenylalanine and tyrosine ($r^2 \geq 0.99$), with a detection limit of 105 nmol/L for Phe and 398 nmol/L for Tyr. Intra-assay and inter-assay coefficients of variation were < 5.3% and < 6.2%, respectively, for the p.R158Q variant in lower tyrosine range. Recovery of tyrosine was 100%. Compared to the wild type enzyme, the highest PAH activity at standard conditions (1 mmol/L Phe; 200 μmol/L BH_4) was found for the mutant p.Y417C (76%), followed by p.E390G (54%), p.R261Q (43%), p.I65T (33%), p.E280A (15%), p.R158Q (5%), and p.R408W (2%). A relative high PAH activity was found in kidney (33% of the liver activity), but none in brain.

Conclusions: This novel method is highly sensitive, specific, reproducible, and efficient, allowing the quantification of PAH activity in different cells or tissue extracts using minimum amounts of samples under standardized conditions.

Introduction

Deficiency of phenylalanine hydroxylase (PAH, EC 1.14.16.1) is causing phenylketonuria (PKU, OMIM 261600), an autosomal recessively inherited disease presenting with elevated blood phenylalanine (Phe) levels [1, 2]. The phenotypic severity of PKU is characterized by the type of mutation, and thus by residual PAH enzyme activity. The fully functional homotetrameric PAH catalyzes hydroxylation of Phe to tyrosine (Tyr) in the presence of cofactor (6*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) and molecular oxygen [3, 4]. According to the Locus Knowledgebase (*PAH*db, www.pahdb.mcgill.ca), about 60% of mutations in the *PAH* gene are missense mutations, which may lead to a misfolding of the protein [5, 6], disturbing the complex enzyme regulation and changes in kinetics, due to altered affinities for the Phe substrate and the BH_4 cofactor.

The incidence of PKU is about 1 in 10,000 newborns in Caucasian populations [2]. For most patients, therapy consists in a life-long dietary restriction of Phe to prevent neurological impairment. Recently, it has been reported that a subgroup of PKU patients (mild to moderate phenotype) can benefit from a pharmacological therapy with BH_4 (sapropterin dihydrochloride; Kuvan®) [7,8]. Newborn screening program for PKU, initially based on the Guthrie test [9], has been established for the early detection of PKU patients. Today, electrospray ionization tandem mass spectrometry (ESI-MSMS or TMS) is the method of choice for fast screening and monitoring of Phe and Tyr levels in dried blood spots (DBS) [10]. Of the over 550 disease-causing mutations listed in the PAHdb [11], 88 were expressed in different *in vitro* cell systems to estimate the residual PAH activity. Expression systems like *Escherichia coli*, eukaryotic cell lines, or cell-free systems were most commonly used systems [12 - 14]. In addition to cell systems, PAH activity was studied in rat liver biopsy samples [15]. Expression of recombinant PAH in bacteria was applied for characterization of physical and chemical properties of the enzyme [16].

Previous methods for PAH activity measurement were based on the determination of ^{14}C-labeled Tyr produced [3] or release of ^{3}H [17]. Other methods are based on detection of Tyr by fluorescence coupled to HPLC [18], colorimetric assays [19], or fluorescence monitoring [20]. Recently described method by Gersting et al. [20] was developed for characterization of purified mutant PAH proteins at different Phe and BH_4 concentrations.

In our novel assay, we applied liquid chromatography (LC) with ESI-MSMS for the quantification of Tyr produced from Phe. Prior to analysis, the amino acids are derivatized to propyl chloroformate derivatives, using the commercially available Phenomenex EZ:faast™

kit. Our method allows for short analysis times and lower limit of detection and is optimized for determination of PAH enzyme activity of recombinantly expressed mutant proteins in COS-1 and other cell lines, as well as in mice liver, kidney, and brain. Thus, it allows comparison between different mutant proteins at standard conditions.

Materials and methods

Materials

The Phenomenex EZ:faast^{TM} kit for LC with ESI-MSMS amino acid analysis was purchased from Phenomenex (Torrance, CA, USA). L-phenylalanine-d_5 and L-tyrosine-d_4 standard reagents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) whereas L-phenylalanine-d_8 was obtained from C/D/N Isotopes Inc. (Pointe Claire, Quebec, Canada). L-Phenylalanine and L-Tyrosine, as well as the DMEM cell culture medium were purchased from Sigma Aldrich (St. Louis, MO, USA). RPMI 1640 medium was from Invitrogen (San Diego, CA, USA). BH_4 dihydrochloride was obtained from Schircks Laboratories (Jona, Switzerland). Mouse tissues were extracted from C57Bl/6 (wild type) or C57Bl/6-*Pah*^{enu2} (PKU) [21] mice strains.

Expression plasmid preparation

The expression plasmid pCMV-FLAG-PAH (Promoter-N-Fusion-PAH) was received as courtesy gift from L. R. Desviat [22]. Mutations in the human *PAH-cDNA* sequence were introduced by site-directed mutagenesis, using QuikChange XL kit from Agilent Technologies (Santa Clara, CA, USA) and confirmed by sequencing analysis using BigDye Terminator Cycle sequencing v1.1 (Applied Biosystems) on an ABI Prism 3100 Sequencer.

Transfection of cells and preparation of mouse tissue

Cell lines (COS-1, Hep3B, Huh-7) were cultured either in DMEM or RPMI1640 (HaCat, lymphoblasts) medium, with appropriate additives, at 37°C under 5% CO_2. One day prior to transfection, COS-1 cells were seeded at 2×10^5 cells/mL in 10-cm dishes. Transfection experiments were performed using Fugene 6 (Roche Diagnostics, Mannheim, Germany) according to manufacturer's recommendations. Hereby, 13 µg of the pCMV-FLAG-PAH plasmid (either wild type or mutant) were co-transfected with 2 µg of pSV-βgal reporter plasmid (Promega, Madison, USA). Transfected cells were harvested after 48 h for determination of PAH activity or flash-frozen in liquid N_2 for storage at -80°C. Transfection efficiency was verified by determining β-galactosidase activity in 5 µl lysate in PAH assay cell lysis buffer (1× PBS pH 7.4, 0.25 M sucrose, complete protease inhibitors

cocktail (Roche)) using in-situ β-galactosidase enzyme assay system (Promega, Madison, USA). PAH activities of wild type and mutants were normalized according to transfection efficiency.

Frozen mouse tissues were lysed in homogenization buffer (10 µl/mg tissue), as described in [23], and homogenized using Qiagen TissueLyser II at 4°C. After centrifugation at 13,000 *g* and 4°C for 30 min, supernatants were kept frozen at -80°C.

PAH activity assay

Cell lysates were prepared and enzyme activity was determined using previously described methods [24]. Briefly, assay conditions included pre-incubation at 25°C with Phe (1 mmol/L) for 4 min, then $Fe(NH_4)_2(SO_4)_2$ (100 µmol/L) was added, and incubation was continued for one more minute. After 5 min total pre-incubation time, BH_4 (200 µmol/L for cell extracts or 75 µmol/L for mouse tissue) was added to start the reaction. Between 2.5 and 20 µl (2 - 165 µg) of total protein lysate extracted from cells or mouse tissue was used for activity measurements. The applied total protein amount depended on lysate type, transfected, non-transfected, or mouse tissue samples. It was generally higher for sample types with low activity where only little amounts of Tyr were produced. Initially, this was determined empirically by measuring a series of increasing total protein amounts for each sample type to determine the measurable linear range. Reaction time was 2 min for mouse tissue lysates and 15 min for cell lysates. Short incubation time reduces any possible chaperone-like effect of BH_4. The amount of Tyr produced was determined by LC with ESI-MSMS (see below).

Protein concentrations of all sample types were determined using Pyrogallol Red protein dye binding assay [25] after completion of PAH assay due to low stability of protein lysates. Specific PAH activities are expressed in mU per mg total protein to account for differences in total protein amount and with mU equal to nmol L-Tyr produced per minute.

Stock solution preparation and calibration

Labeled internal standard stock solutions (10 mmol/L Phe-d_5 and 10 mmol/L Tyr-d_4) were prepared in 50 mmol/L HCl, stored at -20°C, and freshly diluted to working concentrations (see sample preparation). Stock solutions of non-labeled Phe (50 mmol/L) and Tyr (8 mmol/L) were prepared for calibration curves in 50 mmol/L HCl and stored at -20°C.

Working solutions for calibration curves were freshly prepared from the non-labeled stock solutions in H_2O from 100 to 700 µmol/L Phe and 4 to 350 µmol/L Tyr (Table 3.1).

In order to include the matrix effect to the calibration curves, 20 µl of COS-1 non-transfected cell lysate was added to the calibration samples.

Table 3.1: Standards used for the calibration curve of L-Tyr and L-Phe.

Standard	1	2	3	4	5	6	7	8	9	10	11	12	13	14
[Tyr] µmol/L	0	4	8	12	16	20	40	60	80	100	150	200	250	350
[Phe] µmol/L	0	100	150	200	250	300	350	400	450	500	550	600	700	-

Sample preparation and derivatization

Samples were prepared according to the Phenomenex EZ:faast^{TM} kit's manual [26], with the following modifications: prior to amino acid extraction and derivatization, 20 µL of each internal standard solution containing 100 µmol/L Phe-d_5 and 20 µmol/L Tyr-d_4 (in 50 mmol/L HCl) were added to 20 µL of sample lysate. Using the kit's reagents, the amino acids are derivatized with propyl chloroformate resulting in the addition of a propyl formate at the amine moiety and a propyl group at the carboxylic end of the amino acids, respectively. The hydroxy group of Tyr is also derivatized by the addition of a propyl formate group.

Instrumentation

For RP (reversed phase)-HPLC separation of amino acids, a 250 × 2 mm C18 column (Phenomenex EZ:faast^{TM}) was used. The derivatized amino acids were separated using the following program: (i) isocratic flow 75% solvent B for 6 min; (ii) linear gradient from 75% to 95% solvent B (v/v) in 9 min; (iii) linear gradient from 95% to 100% solvent B in 0.1 min; (iv) isocratic flow 100% solvent B for 3 min; (v) linear gradient from 100% to 75% solvent B in 0.1 min; (vi) isocratic flow 75% solvent B for 2 min. Solvents A and B were 10 mmol/L ammonium formate in H_2O and 10 nmol/L ammonium formate in methanol, respectively. Flow rate was 150 µL/min, and the injection volume was 10 µL.

A PerkinElmer SCIEX API 2000 LC-ESI-MSMS system equipped with a PerkinElmer Series 200 autosampler and two PerkinElmer Series 200 micro pumps were used for LC–ESI-MSMS analysis. Amino acids were acquired using the multiple reaction mode (MRM) positive ion mode with the following transitions: 294 → 206 (Phe), 299 → 211 (Phe-d_5), 302 → 214 (Phe-d_8), 396 → 308 (Tyr), and 400 → 312 (Tyr-d_4). The dwell time was 500 ms. Mass spectra were acquired in the time range of 6 to 20 min.

Immunoquantification by Western blot

Five to 50 µg of lysate from activity assay was used for verifying PAH expression in the various cell lines or mouse tissue samples by Western blotting, using commercially available

anti-PAH antibody PH8 (Abcam, Cambridge, UK) and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, CA, USA). Antibody binding was detected by enhanced chemiluminescence (Amersham ECL^{TM}) as described by the manufacturer's instructions (GE Healthcare, CT, USA).

Limit of detection

The limit of detection (LOD) was determined by measuring six blank samples of non-transfected COS-1 cell lysate with very low amounts of Phe and Tyr using the following formula:

LOD = ((mean + 3.3)∗standard deviation) / slope.

The limit of quantification (LOQ) for Phe and Tyr was calculated as follows:

LOQ = ((mean + 10)∗standard deviation) / slope.

Results

Linearity and limit of detection

Figure 3.1 depicts linear regression of calibration standards for Phe and Tyr. Both calibration curves were linear with correlation coefficients of $r^2 \geq 0.99$. The LOD was 105 nmol/L for Phe and 398 nmol/L for Tyr and LOQ 147 nmol/L for Phe and 574 nmol/L for Tyr.

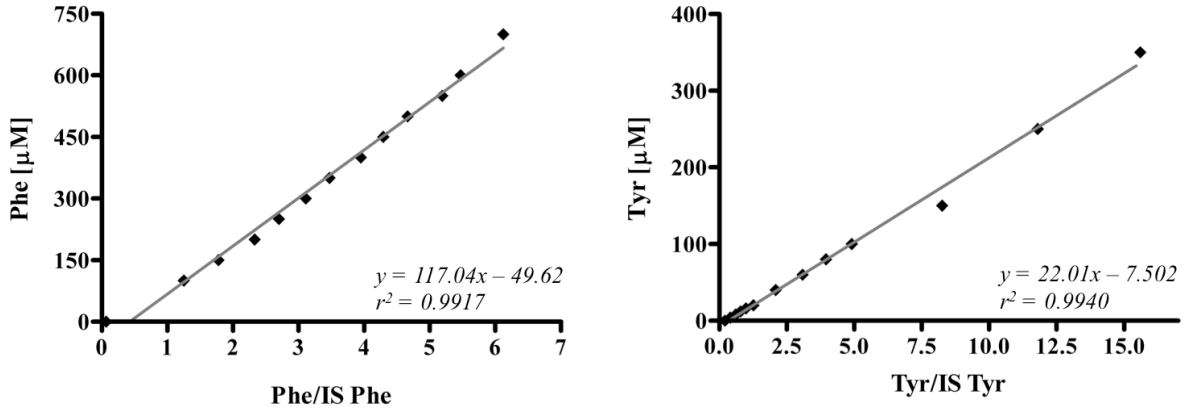


Figure 3.1: Calibration curves (linear regression) for Phe and Tyr using Phe-d_5 and Tyr-d_4 as internal standards for quantification. The ratio of analyte peak area and corresponding internal standards is plotted as a function of analyte concentration.

Imprecision and recovery

Inter- and intra-assay analyses were performed with the wild type enzyme, with medium activity (p.R261Q), and low activity (p.R158Q) PAH variants, transfected into COS-1 cells.

Six samples were each prepared either on the same day (intra-assay) or during the period of two weeks (inter-assay). Table 3.2 summarizes the intra- and inter-assay imprecision measurements of the method.

The intra-assay coefficient of variation (CV) varied between 2.4% and 10.8% and between 2.7% and 8.9% for Tyr and Phe, respectively (Table 3.2). The inter-assay imprecision data for Tyr and Phe ranges from 5.3% to 14.3% (Table 3.2). Comparable CVs in the intra- and inter-assays show that the PAH assay samples are stable over more than 2 weeks at -20°C.

The recovery of 100 µmol/L exogenous Tyr added to the variant PAH p.R261Q after PAH assay was 100% (data not shown).

Analytical performance

Three characteristic parameters, retention time, mother ion mass, and daughter ion mass were measured in the LC-ESI-MSMS method for each analyte, e.g. 12.59 min, m/z 396 Da and m/z 308 for Tyr. Figure 3.2A shows a representative chromatogram of a wild type PAH assay sample with the peak-pairs at 11.0 min representing Phe (294 → 206) and IS-Phe (299→ 211) and at 12.6 min assigned to Tyr (396 → 308) and IS-Tyr (400 → 312). The peaks at 9.0 and 12.2 min correspond to d_3-methionine and homophenylalanine and are the internal standards included in the Phenomenex EZ:faast^{TM} kit.

Table 3.2: Imprecision data of Phe and Tyr quantification determined with wild type, p.R261Q and p.R158Q PAH samples. The intra- and inter-assays were performed with individual cell lysates (n = 6). All PAH activities are normalized by co-transfection with pSV-ßgal.

	Wt PAH		p.R261Q		p.R158Q	
	Mean (SD) µmol/L	CV[a] (%)	Mean (SD) µmol/L	CV[a] (%)	Mean (SD) µmol/L	CV[a] (%)
Intra-assay (n = 6)						
Phe	417.3 (37.2)	8.92	339.8 (9.1)	2.68	436.4 (28.9)	6.63
Tyr	83.2 (9.0)	10.81	142.2 (3.4)	2.38	7.7 (0.5)	6.16
Inter-assay (n = 6)						
Phe	321.5 (18.5)	5.75	414.9 (48.5)	11.68	520.1 (45.0)	8.66
Tyr	210.4 (25.5)	12.13	125.6 (17.9)	14.29	14.9 (0.9)	5.28
PAH activity (mU/mg protein)						
Intra-assay (n = 6)	61.4		16.6		2.34	
Inter-assay (n = 6)	28.5[b]		20.8		2.71	

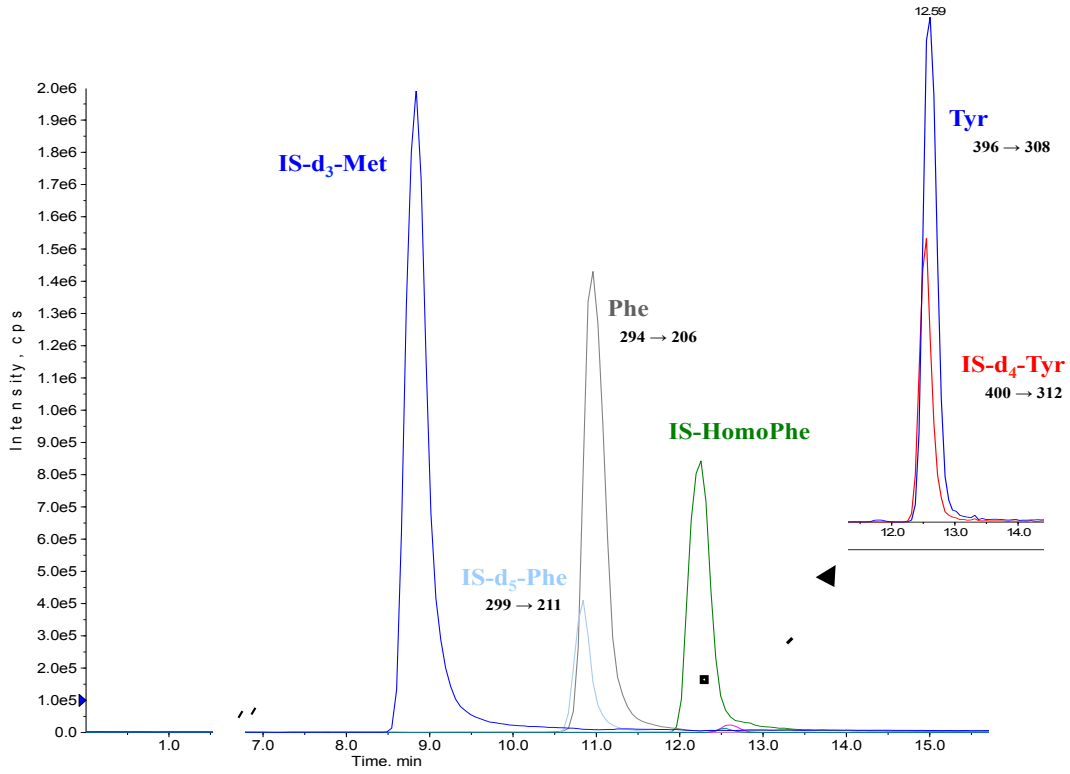
[a] CV = coefficient of variation.
[b] Varying reaction conditions: 30 min reaction time and 24.5 µg total protein in inter-assay compared to 15 min reaction time and 9.0 µg total protein in intra-assay experiment

PAH activity assay

The linearity of the PAH enzyme assay was investigated using transfected COS-1 cells with human wild type PAH. The reaction time was optimized to 15 min. As demonstrated in Figure 3.2B, Tyr production increased linearly with the amount of protein. However, the

linear range of assay is rather small (< 250 µmol/L) and PAH activity decreases with increasing Tyr production, due to reagent availability in the reaction mixture and possible product inhibition.

(A)



(B)

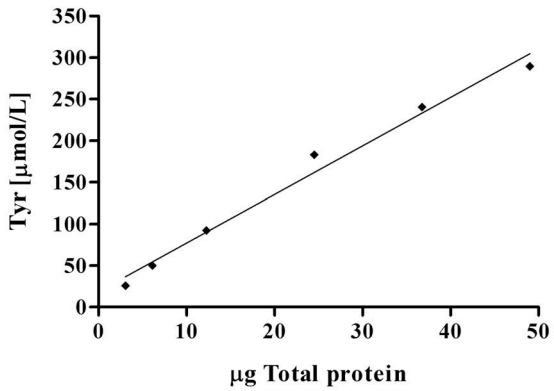


Figure 3.2: (A) Mass spectrum of wild type PAH expressed in COS-1 cells, prepared with EZ:faast kit and measured by LC ESI-MSMS. Preparation by the kit includes 3 additional internal standards to correct for sample preparation variability. These are d_3-methionine, homophenylalanine and homoarginine (not shown in the spectrum above); (B) Linearity of Tyr production from PAH wild type expressed in COS-1 cells transfected with increasing amounts of plasmid.

All mutants produced lower amounts of Tyr at similar reaction times, compared with the wild type enzyme. Stability of PAH during the reaction is critical and short reaction time should be used at low protein concentrations (< 30 µg for the wild type PAH and < 165 µg for low-activity mutants). The mean wild type PAH activity in COS-1 cells was 43.9 ± 14.5 mU/mg protein under standard reaction conditions (15 min reaction time and 5 µl cell lysate).

PAH intra-assay variation determined in triplicate was 7.3%, 6.4%, and 7.5% for the wild type, p.R261Q and p.I65T in COS-1 cells, respectively. Comparative analyses of activity for wild type PAH activity in transfected COS-1 cells using a HPLC-based assay with fluorimetric detection [27] provided similar values of activity for wild type (38 ± 2 mU/mg) and p.R261Q mutant (16 ± 1 mU/mg). However, this HPLC based analysis requires > 30 µg of protein in each assay, i.e. 1.5–6 fold higher than in the LC-ESI-MSMS assay at the selected conditions.

PAH mutations found in PKU patients

In order to further evaluate the LC-ESI-MSMS method, we determined PAH activity of several known PAH mutations, in addition to p.R261Q and p.R158Q used in the method characterization and development (see above). Figure 3.3A shows the percent of PAH mutant activities compared to the wild type activity for the p.I65T (33%), p.R261Q (43%), p.R158Q (5%), p.R408W (2%) and p.E390G (54%) proteins.

These mutations have previously been tested in different *in vitro* systems, but we also analyzed two novel PAH mutations p.E280A and p.Y417C, recently reported in Turkish PKU patients [28] (Figure 3.3A). While a severe p.E280A mutation exhibits only 15% of the wild type PAH activity, a mild p.Y417C mutation was almost as active as the wild type enzyme (76%). All mutant PAH activities were normalized for β-galactosidase, which was co-transfected into COS-1 cells. Figure 3.3B illustrates the different PAH mutant proteins by Western blot analysis.

All of the PAH mutant proteins with rather low activity also present with reduced amounts of PAH protein, when compared to the wild type (Figure 3.3C). The only exception is p.R158Q with a very low activity and substantial amount of protein. PAH expression was normalized for β-actin expression in these COS-1 cells and quantified by densitometry.

PAH activity in mouse tissue

The quantification of PAH activity with the novel LC–ESI-MSMS method is not only restricted to cell extracts, but can also be used for animal tissues. For this purpose, we measured PAH activity in liver, kidney, and brain tissue from wild type (C57Bl/6) or PKU mice (C57Bl/6-*Pah*^{enu2}). Figure 3.4A shows PAH activity in liver, kidney and brain extracts from the wild type and PKU mice. As expected, the highest activity was found in liver of the wild type mouse. A relatively high PAH activity (33% of the liver) was found in the kidney, whereas in tissues from the PKU mice no PAH activity was detectable. No PAH activity was observed in brain extracts from the wild type and PKU animals.

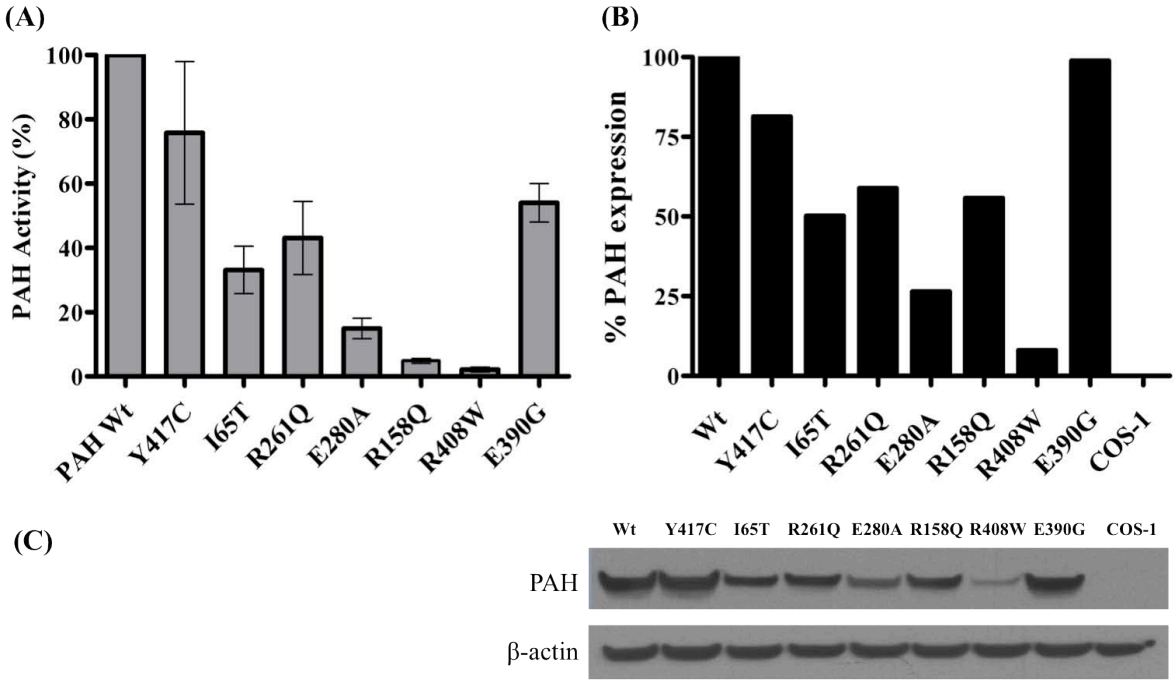


Figure 3.3: (A) PAH activities of various common PAH mutant proteins expressed in COS-1 cells and quantified by LC-ESI-MSMS. Activities are displayed as % wt activity. All PAH activities are normalized with β-galactosidase activity for correction of transfection efficiency. (B) Western blot analysis of wild type PAH and mutations tested for PAH activity. The same amount of total protein (25 µg) was analyzed per lane. (C) PAH expression in all samples was normalized with β-actin expression and expressed as % wild type PAH expression. Non-transfected COS-1 cells do not show PAH protein.

The Western blot analysis shows high amounts of PAH protein in the wild type mice extracts from liver and kidney, while in the same organs of the PKU mice bands of the PAH protein were detected despite no activity (Figure 3.4B). These results correlate well with the enzyme activity measurements for the wild type mouse (Figure 3.4A).

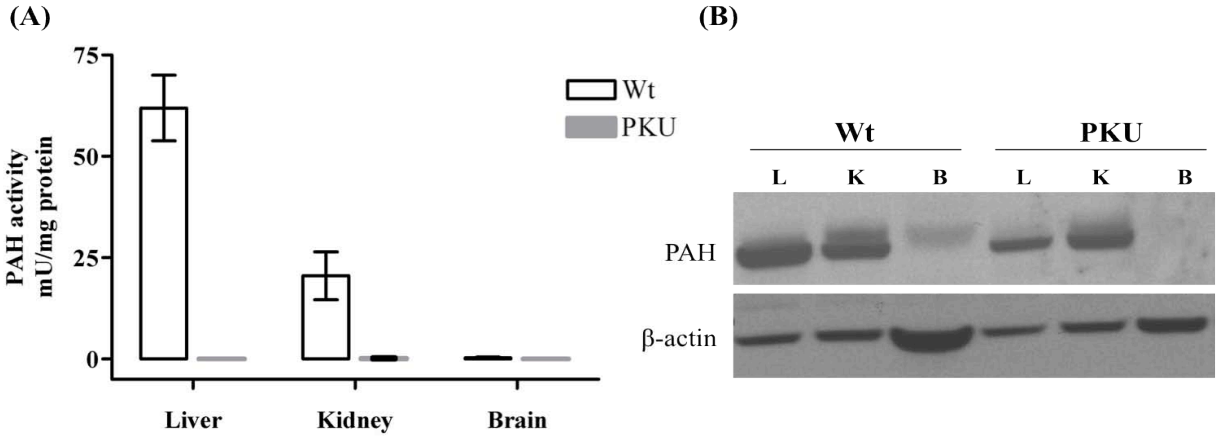


Figure 3.4: (A) Comparison of absolute PAH activity in mouse tissues of wild type (C57Bl/6) and PKU (C57Bl/6-*Pah*^{enu2}) mice (n = 3). Standard deviation results for PKU liver and brain are not displayed due to very small values. (B) Western blot analysis of PAH expression in liver (L), kidney (K) and brain (B) tissue from wild type and PKU mice. As shown by β-actin loading control, increased protein amounts of brain tissue were loaded.

The PAH expression seems to appear as two signals in Western blot analysis. This double band of PAH has previously been observed for PAH in tissues and when expressed in eukaryote systems, and was found to be the result of an apparent electrophoretic shift of the Ser-16 phosphorylated enzyme [4].

PAH activity in different cell lines

For *in vitro* PAH expression studies, liver, or kidney mammalian cells (organs with highest PAH activity) exhibit the most similar physiological environment. However, these cell lines might exhibit endogenous PAH activity that interfere with activity from transfected PAH. Therefore, we investigated PAH expression and PAH activity in several common hepatic cell lines (e.g. HepG2, Hep3B and HuH-7) (Figure 3.5A). No PAH activity or protein was detected in HepG2 cells, and only low endogenous activity was found in Hep3B and HuH-7 cells (Figures 3.5A and B).

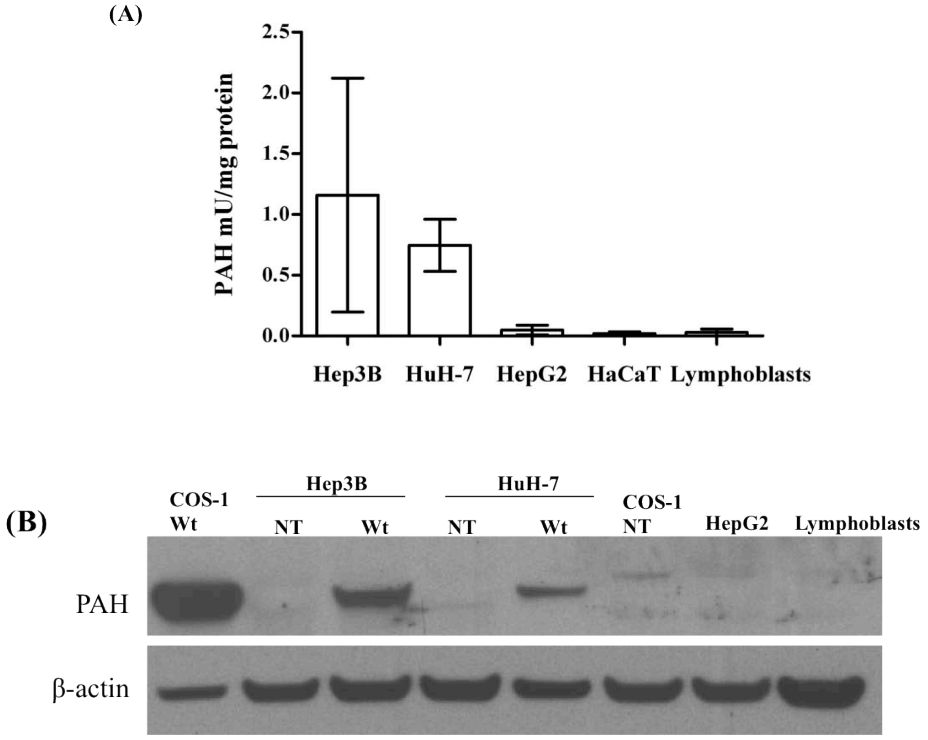


Figure 3.5: (A) PAH activity in various cell lines, eventually for use as cell host in *in vitro* studies or previously analyzed in PAH metabolism. (B) Western blot analysis of protein lysates from various cell lines transfected with wild type PAH probed for PAH expression. 20µg of total protein analyzed (5 µg of COS-1 wild type due to high expression), NT = non-transfected

Using the labeled Phe-d_8 isotope in the PAH assay, which is consequently oxidized to Tyr-d_7, we were able to distinguish the assay product Tyr-d_7 from Tyr from the cell extracts. We found that PAH activities using Phe-d_8 are lower than using non-labeled Phe (32.5 mU/mg versus 43.7 mU/mg protein) due to a possible isotope effect, but high enough to discriminate between different PAH mutants. The same reduction accounts for the p.R261Q

mutant, but the activity of this mutant is consistently measured to be 40% of wild type PAH activity both with Phe or Phe-d_8 as substrates.

Figure 3.5B shows wild type PAH protein expression in Hep3B, HuH-7, and COS-1 cells, compared to non-transfected cells. No wild type PAH protein could be detected from non-transfected Hep3B, HuH-7, and COS-1 cell lines with 20 µg of total protein analyzed.

Furthermore, no PAH activity or protein was detected in EBV-transformed lymphocytes, even though *PAH* transcript can be amplified from their RNA [29]. Also, no PAH activity and transcript could be detected in the HaCaT cell line (human adult low calcium high temperature keratinocytes).

Discussion

Molecular mechanisms of PKU and other hyperphenylalaninemias were established over several decades through investigations of mutations within the PAH gene [30]. Based on genotype findings and description of BH_4-responsive forms of PKU [31], functional assays of mutation effects *in vitro* have proven to be very fruitful for the characterization of PAH mutations, building a bridge between patient and pure protein. Although, these data sets tend to overestimate PAH activities *in vivo*. Residual *in vitro* PAH activity was shown to correlate with the patient's phenotype [32]. Thus, knowing patient's residual PAH activity can be relevant for selecting therapeutic options, the likely Phe tolerance, and the expected response to BH_4 [28].

In this study, we have developed a new method for the quantification of the amino acids Phe and Tyr by LC-ESI-MSMS, as a basis for the measurement of PAH activity. The EZ:faast^{TM} sample preparation (amino acid extraction, washing and derivatization steps are included in the procedure) was originally developed for the rapid processing and measuring of biological fluids in clinical analyses. We optimized this procedure to the quantitative determination of PAH activity in an *in vitro* expression system and in various mouse tissues. Using the EZ:faast^{TM} kit, sample preparation is fast and time consuming steps, such as protein precipitation, removal of interfering substances (e.g. salts and other buffer components), or elaborating derivatization procedures, can be omitted. Furthermore, since our mass spectrometric analysis technique enables the application of isotopically labeled internal standards (Phe-d_5 and Phe-d_8, Tyr-d_4), our method is highly specific and reliable. It is suitable for the quantification of Phe and Tyr even in very complex biological matrices. Its fast and simple sample preparation procedure allows for the analysis of a big sample series in a short amount of time.

The calibration range of Phe and Tyr is variable and can be selected according to the type of *in vitro* expression system and predicted enzyme activity. Low coefficients of variation in intra- and inter-assay confirm that the method can be applied in the nanomolar range.

The PAH assay method used for the quantification by MS is a discontinuous assay commonly used in PAH mutation studies. The time between extraction of total protein from cultured cells and enzymatic reaction is critical and should be kept short. Our expression system in COS-1 cells shows a high transfection efficiency and protein expression, therefore only little amounts of total protein are required for assessing activity. We verified this method to determine activities in the linear range and diluted the proteins accordingly. Thus, other systems with lower protein expression can be easily quantified by this new method.

Every PAH mutant was assessed in at least three different transfection systems and quantification experiments. The results of each mutant, expressed as percent of wild type PAH, are well in accordance with previously published data for the same mutations [33]. The slight differences found were due to the fact that previously reported PAH activity measurements were performed under different assay conditions and using several substrates with different affinities (e.g. BH_4, 6-methyl-tetrahydropterin or 6,7-dimethyl-tetrahydropterin) [3, 12]. In previous reports, *in vitro* activities for some mutations (e.g. p.R261Q) ranged between 24 and 100%, depending on the expression system and assay used [34, 35].

Two new PKU mutations, p.E280A and p.Y417C, are described in our study, which were detected among a larger Turkish patients cohort and were not previously expressed *in vitro*. The activities determined for p.E280A and p.Y417C mutants (15% and 76% of the wild type PAH) correlate with the severity of hyperphenylalaninemia in the corresponding patients.

The quantification of Phe and Tyr is not only limited to cell extracts, but can also be used on various mouse tissues. The advantages of using Phenomenex EZ:faast^{TM} kit also apply to these complex biological samples, omitting interferences from the mixtures. Thus, the liver is not the only site of PAH expression in humans and animals. Up to 40% PAH activity has been reported in the kidneys, as compared to the liver in human tissue [36]. In this study, we report 33% of kidney PAH activity in wild type mouse tissue in relation to PAH activity in liver, in accordance with previous data in rodents [37]. In addition to liver and kidneys, significant PAH activity and expression were also reported in pancreas tissue [36, 38]. Keratinocytes were suggested as potential target for PKU gene therapy [39] and claimed to contain intact PAH metabolism [40], however with our assay no PAH activity or protein was detected in these cells.

In contrast to the method published by Gersting et al. [20], our method measures PAH activity at standardized Phe and BH_4 concentrations and may be a good screening method for selection of mutations of interest for the kinetic studies at different Phe and BH_4 concentrations [41]. Thus, the main application of this new method will be the quantification of the relative *in vitro* PAH activity in patients with PKU by expressing mutant proteins in COS-1 cells. A recent study with a large cohort of Turkish PKU patients suggested that both the phenotype and BH_4 responsiveness are genotype-dependent and that the residual PAH activity is a good predictor for the severity of the disease [28]. Finally, this method can easily be extended to other amino acids since labeled compounds are available as internal standards for quantification.

Acknowledgments

This work was supported by the Swiss National Science Foundation grant no. 31003A-119982 (to NB and BT). We would like to thank Dr. L. Ruiz Desviat for providing us with the PAH expression plasmid and Dr. H. M. Viecelli for the mouse tissue samples and Dr. R. Dummer for keratinocytes. In addition, we thank the mass spectrometry group of the Children's Hospital in Zurich for expert technical assistance.

References

[1] C.R. Scriver, S. Kaufman, Hyperphenylalaninemia: phenylalanine hydroxylase deficiency, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, B. Vogelstein (Eds.), The Metabolic and Molecular Bases of Inherited Disease, 8th ed., McGraw-Hill, New York, 2001, pp. 1667–1724.

[2] N. Blau, F.J. Van Spronsen, H.L. Levy, Phenylketonuria, Lancet 376 (2010) 1417–1427.

[3] S. Kaufman, The phenylalanine hydroxylating system, Adv. Enzymol. Relat. Areas Mol. Biol. 67 (1993) 77–264.

[4] E. Bjorgo, P.M. Knappskog, A. Martinez, R.C. Stevens, T. Flatmark, Partial characterization and three-dimensional-structural localization of eight mutations in exon 7 of the human phenylalanine hydroxylase gene associated with phenylketonuria, Eur. J. Biochem. 257 (1) (1998) 1–10.

[5] A.L. Pey, F. Stricher, L. Serrano, A. Martinez, Predicted effects of missense mutations on native-state stability account for phenotypic outcome in phenylketonuria, a paradigm of misfolding diseases, Am. J. Hum. Genet. 81 (5) (2007) 1006–1024.

[6] S.W. Gersting, K.F. Kemter, M. Staudigl, D.D. Messing, M.K. Danecka, F.B. Lagler, C.P. Sommerhoff, A.A. Roscher, A.C. Muntau, Loss of function in phenylketonuria is caused by impaired molecular motions and conformational instability, Am. J. Hum. Genet. 83 (1) (2008) 5–17.

[7] A.C. Muntau, W. Röschinger, M. Habich, H. Demmelmair, B. Hoffmann, C.P. Sommerhoff, A.A. Roscher, Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria, N. Engl. J. Med. 347 (2002) 2122–2132.

[8] N. Blau, Sapropterin dihydrochloride for phenylketonuria and tetrahydrobiopterin deficiency, Expert. Rev. Endocrinol. Metab. 5 (4) (2010) 483–494.

[9] R. Guthrie, A. Susi, A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants, Pediatrics 32 (1963) 338–343.

[10] D.H. Chace, D.S. Millington, N. Terada, S.G. Kahler, C.R. Roe, L.F. Hofman, Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry, Clin. Chem. 39 (1) (1993) 66–71.

[11] C.R. Scriver, P.J. Waters, C. Sarkissian, S. Ryan, L. Prevost, D. Cote, J. Novak, S. Teebi, P.M. Nowacki, PAHdb: a locus-specific knowledgebase, Hum. Mutat. 15 (1) (2000) 99–104.

[12] P.J. Waters, M.A. Parniak, P. Nowacki, C.R. Scriver, In vitro expression analysis of mutations in phenylalanine hydroxylase: linking genotype to phenotype and structure to function, Hum. Mutat. 11 (1) (1998) 4–17.

[13] S.C. Kwok, F.D. Ledley, A.G. DiLella, K.J. Robson, S.L. Woo, Nucleotide sequence of a full-length complementary DNA clone and amino acid sequence of human phenylalanine hydroxylase, Biochemistry 24 (3) (1985) 556–561.

[14] F.D. Ledley, H.E. Grenett, A.G. DiLella, S.C. Kwok, S.L. Woo, Gene transfer and expression of human phenylalanine hydroxylase, Science 228 (4695) (1985) 77–79.

[15] K.H. Nielsen, Rat liver phenylalanine hydroxylase. A method for the measurement of activity, with particular reference to the distinctive features of the enzyme and the pteridine cofactor, Eur. J. Biochem., FEBS 7 (3) (1969) 360–369.

[16] A. Martinez, P.M. Knappskog, S. Olafsdottir, A.P. Doskeland, H.G. Eiken, R.M. Svebak, M. Bozzini, J. Apold, T. Flatmark, Expression of recombinant human phenylalanine hydroxylase as fusion protein in Escherichia coli circumvents proteolytic degradation by host cell proteases. Isolation and characterization of the wild-type enzyme, Biochem. J. 306 (Pt 2) (1995) 589–597.

[17] G. Guroff, C.A. Rhoads, A. Abramowitz, A simple radioisotope assay for phenylalanine hydroxylase cofactor, Anal. Biochem. 21 (2) (1967) 273–278.

[18] S.W. Bailey, J.E. Ayling, An assay for picomole levels of tyrosine and related phenols and its application to the measurement of phenylalanine hydroxylase activity, Anal. Biochem. 107 (1) (1980) 156–164.

[19] S. Udenfriend, J.R. Cooper, The chemical estimation of tyrosine and tyramine, J. Biol. Chem. 196 (1) (1952) 227–233.

[20] S.W. Gersting, M. Staudigl, M.S. Truger, D.D. Messing, M.K. Danecka, C.P. Sommerhoff, K.F. Kemter, A.C. Muntau, Activation of phenylalanine hydroxylase induces positive cooperativity towards the enzyme's natural cofactor, J. Biol. Chem. 285 (2010) 30686–30697.

[21] Z. Ding, P. Georgiev, B. Thony, Administration-route and gender-independent long-term therapeutic correction of phenylketonuria (PKU) in a mouse model by recombinant adeno-associated virus 8 pseudotyped vector-mediated gene transfer Gene Ther 13 (2006) 587–593.

[22] C. Aguado, B. Perez, M. Ugarte, L.R. Desviat, Analysis of the effect of tetrahydrobiopterin on PAH gene expression in hepatoma cells, FEBS Lett. 580 (7) (2006) 1697–1701.

[23] L. Elzaouk, W. Leimbacher, M. Turri, B. Ledermann, K. Bürki, N. Blau, B. Thöny, Dwarfism and low IGF-1 due to dopamine depletion in Pts−/− mice rescued by feeding neurotransmitter precursors and H4-biopterin, J. Biol. Chem. 278 (2003) 28303–28311.

[24] A.L. Pey, M. Ying, N. Cremades, A. Velazquez-Campoy, T. Scherer, B. Thony, J. Sancho, A. Martinez, Identification of pharmacological chaperones as potential therapeutic agents to treat phenylketonuria, J. Clin. Invest. 118 (8) (2008) 2858–2867.

[25] N. Watanabe, S. Kamei, A. Ohkubo, M. Yamanaka, S. Ohsawa, K. Makino, K. Tokuda, Urinary protein as measured with a pyrogallol red-molybdate complex, manually and in a Hitachi 726 automated analyzer, Clin. Chem. 32 (8) (1986) 1551–1554.

[26] Phenomenex, Ez:Faast (Easy–Fast) Amino Acid Sample Testing Kit Manual, Phenomenex, Torrance, CA, 2001.

[27] A.P. Doskeland, S.O. Doskeland, D. Ogreid, T. Flatmark, The effect of ligands of phenylalanine 4-monooxygenase on the cAMP-dependent phosphorylation of the enzyme, J. Biol. Chem. 259 (18) (1984) 11242–11248.

[28] S.F. Dobrowolski, C. Heintz, T. Miller, C.R. Ellingson, C.C. Ellingson, I. Özer, G. Gökcay, T. Baykal, B. Thöny, M. Demirkol, N. Blau, Molecular genetics and impact of residual in vitro phenylalanine hydroxylase activity on tetrahydrobiopterin-responsiveness in Turkish PKU population, Mol. Genet. Metab. 10 (2011) 116–121.

[29] S.J. Ramus, S.M. Forrest, R.G. Cotton, Illegitimate transcription of phenylalanine hydroxylase for detection of mutations in patients with phenylketonuria, Hum. Mutat. 1 (2) (1992) 154–158.

[30] F. Güttler, P. Guldberg, Mutations in the phenylalanine hydroxylase gene: genetic determinants for the phenotypic variability of hyperphenylalaninemia, Acta Paediatr. Suppl. 407 (1994) 49–56.

[31] S. Kure, D.C. Hou, T. Ohura, H. Iwamoto, S. Suzuki, N. Sugiyama, O. Sakamoto, K. Fujii, Y. Matsubara, K. Narisawa, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, J. Pediatr. 135 (3) (1999) 375–378.

[32] Y. Okano, R.C. Eisensmith, F. Güttler, U. Lichter-Konecki, D.S. Konecki, F.K. Trefz, M. Dasovich, T. Wang, K. Henriksen, H. Lou, et al., Molecular basis of phenotypic heterogeneity in phenylketonuria, N. Engl. J. Med. 324 (18) (1991) 1232–1238.

[33] C.R. Scriver, M. Hurtubise, D. Konecki, M. Phommarinh, L. Prevost, H. Erlandsen, R. Stevens, P.J. Waters, S. Ryan, D. McDonald, C. Sarkissian, PAHdb 2003: what a locus-specific knowledgebase can do, Hum. Mutat. 21 (4) (2003) 333–344.

[34] Y. Okano, T. Wang, R.C. Eisensmith, F. Guttler, S.L. Woo, Recurrent mutation in the human phenylalanine hydroxylase gene, Am. J. Hum. Genet. 46 (5) (1990) 919–924.

[35] T. Gjetting, M. Petersen, P. Guldberg, F. Guttler, In vitro expression of 34 naturally occurring mutant variants of phenylalanine hydroxylase: correlation with metabolic phenotypes and susceptibility toward protein aggregation, Mol. Genet. Metab. 72 (2) (2001) 132–143.

[36] U. Lichter-Konecki, C.M. Hipke, D.S. Konecki, Human phenylalanine hydroxylase gene expression in kidney and other nonhepatic tissues, Mol. Genet. Metab. 67 (4) (1999) 308–316.

[37] M.C. Hsieh, H.K. Berry, Distribution of phenylalanine hydroxylase (EC 1.14.3.1) in liver and kidney of vertebrates, J Exp Zool 208 (2) (1979) 161–167.

[38] A. Tourian, J. Goddard, T.T. Puck, Phenylalanine hydroxylase activity in mammalian cells, J. Cell. Physiol. 73 (2) (1969) 159–170.

[39] R. Christensen, F. Guttler, T.G. Jensen, Comparison of epidermal keratinocytes and dermal fibroblasts as potential target cells for somatic gene therapy of phenylketonuria, Mol. Genet. Metab. 76 (4) (2002) 313–318.

[40] K.U. Schallreuter, M. Zschiesche, J. Moore, A. Panske, N.A. Hibberts, F.H. Herrmann, H.R. Metelmann, J. Sawatzki, In vivo evidence for compromised phenylalanine metabolism in vitiligo, Biochem. Biophys. Res. Commun. 243 (2) (1998) 395–399.

[41] M. Staudigl, S.W. Gersting, M.K. Danecka, D.D. Messing, M. Woidy, D. Pinkas, K.F. Kemter, N. Blau, A.C. Muntau, The interplay between genotype, metabolic state, and cofactor treatment governs phenylalanine hydroxylase function and drug response, Hum. Mol. Genet. 20 (2011) 2628–2641.

Chapter 4

Splicing of Phenylalanine Hydroxylase (*PAH*) Exon 11 is Vulnerable: Molecular Pathology of Mutations in *PAH* Exon 11

Caroline Heintz[1], Steven F. Dobrowolski[2], Henriette Skovgaard Andersen[3], Mübeccel Demirkol[4], Nenad Blau[1,5,6,7]* and Brage Storstein Andresen[3]*

[1]*Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Zurich, Switzerland*

[2]*Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA*

[3]*Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense M, Denmark*

[4]*Istanbul University, Istanbul Faculty of Medicine, Children's Hospital, Division Nutrition and Metabolism, Istanbul, Turkey*

[5]*Zürich Center for Integrative Human Physiology (ZIHP), Zürich, Switzerland*

[6]*Research Center for Children (RCC), Zürich, Switzerland*

[7]*Division of Inborn Errors of Metabolism, University Children's Hospital Heidelberg, Germany*

Published in Molecular Genetics and Metabolism 2012, in press.

Abstract

In about 20 - 30% of phenylketonuria (PKU) patients, phenylalanine (Phe) levels can be controlled by cofactor 6*R*-tetrahydrobiopterin (BH_4) administration. The phenylalanine hydroxylase (*PAH)* genotype has predictive value concerning BH_4-response and therefore a correct assessment of the mutation molecular pathology is important. Mutations that disturb the splicing of exons (e.g. interplay between splice site strength and regulatory sequences like exon splicing enhancers (ESEs)/exon splicing silencers (ESSs) may cause different severity of PKU. In this study, we identified *PAH* exon 11 as a vulnerable exon and used patient derived lymphoblast cell lines and *PAH* minigenes to study the molecular defect that impacted pre-mRNA processing. We showed that the c.1144T>C and c.1066-3C>T mutations cause exon 11 skipping, while the c.1139C>T mutation is neutral or slightly beneficial. The c.1144T>C mutation resides in a putative splicing enhancer motif and binding by splicing factors SF2/ASF, SRp20 and SRp40 is disturbed. Additional mutations in potential splicing factor binding sites contributed to elucidate the pathogenesis of mutations in *PAH* exon 11.

We suggest that *PAH* exon 11 is vulnerable due to a weak 3' splice site and that this makes exon 11 inclusion dependent on an ESE spanning position c.1144. Importantly, this implies that other mutations in exon 11 may affect splicing, since splicing is often determined by a fine balance between several positive and negative splicing regulatory elements distributed throughout the exon.

Exonic mutations that disrupt splicing are unlikely to facilitate response to BH_4 and may lead to inconsistent genotype-phenotype correlations. Therefore, recognizing such mutations enhances our ability to predict the BH_4-response.

Introduction

Hyperphenylalaninemia (HPA) is the result of a defect in hydroxylation of phenylalanine (Phe) to tyrosine (Tyr) [1]. The reaction is catalyzed by phenylalanine hydroxylase (PAH, EC 1.14.16.1) requiring the essential cofactor tetrahydrobiopterin (BH_4) [2]. In the majority of cases hyperphenylalaninemia (HPA) is caused by mutations in the *PAH* gene, resulting in different phenotypes classified according to Phe levels in the blood ranging from mild HPA, mild PKU to classic PKU. PKU is a very heterogeneous disease and belongs to the most common inherited diseases in amino acid metabolism [3]. As elevated Phe levels cause severe brain damage, it is compulsory to start treatment as early as possible.

Over 500 mutations have been reported in the coding sequence as well as in the intervening sequence of the *PAH* gene (Online database, http://www.pahdb.mcgill.ca/) [4]. More than half of these are classified as missense changes. Several PKU mutations have been shown to affect protein folding, thereby causing accelerated degradation and/or aggregation [5]. The measurement of enzymatic activities *in vitro* of mutant proteins can generally be useful in predicting HPA's, but it has also been suggested that up to 50% of exonic mutations may perturb pre-mRNA splicing, thereby leading to more deleterious effects on protein function, irrespective of the predicted amino acid change [6].

In ~ 30% of PKU patients (all phenotypes), Phe levels may be controlled through BH_4 (sapropterin dihydrochloride [7]) therapy [8]. Only the patient's full genotype determines BH_4 responsiveness [9, 10], but genotype-phenotype correlations are not always reliable as discordant results have been observed between patients with common genotypes [11]. Exonic mutations that disrupt splicing are unlikely to facilitate BH_4-response and recognizing such mutations enhances our ability to predict BH_4 responsiveness. It is therefore important to correctly assess the molecular pathology of *PAH* mutations.

Cis-acting elements such as exon splicing enhancers (ESE) or exon splicing silencers (ESS) participate in exon recognition in a finely balanced interplay with splice site strengths and this fine balance can be disturbed through deleterious effects of mutations in these elements. When bound to ESEs serine/arginine-rich proteins (SR proteins) promote exon definition by directly recruiting the splicing machinery through their RS domain and/or by antagonizing the action of nearby silencer elements [12].

Relatively few studies have investigated mRNA processing defects owing to exonic sequence variation in the *PAH* gene. The mutation c.611A>G, putatively p.Y204C, was investigated for a role in mRNA processing when *in vitro* assessment of the mutant enzyme

did not demonstrate significantly reduced activity and correspond to the phenotype in the PKU patients [13]. Analysis of the *PAH* mRNA in a patient lymphoblast cell line showed that the c.611A>G mutation masquerades as a missense mutation, but actually creates a new 5' splice site resulting in a 96nt deletion at the 3' end of *PAH* exon 6. This study, together, with the finding that a synonymous mutation, c.1197A>T, causes exon 11 skipping instead of being neutral [14] and our recent study of a c.30C>G synonymous mutation, which creates an ESS [15] with unexpected effects on mRNA splicing, show that more detailed analysis of *PAH* pre-mRNA processing may be required to determine a mutation's molecular pathology which ultimately may relate to both the patient's phenotype and the possibility of BH_4-response. Herein, we established and validated a *PAH* exon 11 minigene, which allows testing the impact of *PAH* exon 11 missense and splice site mutations on mRNA splicing. Both natural mutations and several artificial mutations were investigated to gain insight into the splicing mechanism of *PAH* exon 11. The pathology of the two exonic mutations, c.1139C>T and c.1144T>C, was analyzed by transfection of the minigene reporter, by RNA affinity purification and results were confirmed by analysis of patient cell lines.

Materials and Methods

Patient specimens

Patient samples analyzed in this work were previously reported among a large cohort of Turkish PKU patients [10]. Table 4.1 summarizes the genotypes and phenotypes of the 4 patients analyzed in this study. Patients 1 and 2 have been identified as BH_4-responsive by the oral loading test.

Table 4.1: Summary of genotype and phenotype of PKU patients analyzed in this work.

Genotype	Variation	Phenotype
1. c.1066-3C>T/c.1066-3C>T	IVS10-3C>T/IVS10-3C>T	Mild HPA
2. c.1066-3C>T/c.1208C>T	IVS10-3C>T/p.A403V	Mild HPA
3. c.1144T>C/c.1144T>C	p.F382L/p.F382L	Mild HPA
4. c.1139C>T/c.898G>T	p.T380M/p.A300S	Mild HPA

Generation of patient cell lines and cell culture

Peripheral blood lymphocytes from PAH deficient patients were transformed with Epstein-Barr virus [16] to generate lymphoblast cell lines. Lymphoblast cell lines and Chang human liver epithelial cells were cultured in RPMI 1640 (Sigma Aldrich, St. Louis, MO, USA) and 5% fetal calf serum. COS-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10% fetal calf serum.

To perform nonsense-mediated mRNA decay analysis, lymphoblast cells were cultured overnight in presence of 10 µg cycloheximide (Sigma) prior to mRNA extraction.

Minigene construction

Initially, a 1946-bp fragment of human *PAH* including exon 10, intron 10, exon 11, intron 11 reduced to 988bp and exon 12 was synthesized by GenScript (NJ, USA). A start codon was added to exon 10 and a *KpnI* site was removed from intron 10 to facilitate cloning. In addition, intron 11 was extended by inserting at an *EcoRI* site a PCR amplified 963 bp fragment amplified using AccuPrime Pfx SuperMix (Invitrogen). Five unique minigene constructs were prepared: WT, c.1139C>T, c.1144T>C, c.1066-3C>T and c.1197A>T. The minigenes were cloned into the polylinker of pcDNA3.1+ vector (Invitrogen) by using *KpnI* and *XhoI* restriction enzymes. The correct insertion was verified by sequencing with BigDye Terminator Cycle sequencing v1.1 (Applied Biosystems) on an ABI Prism 3100 Sequencer.

Other mutations used for the characterization of eventual splicing regulatory elements caused by the mutations of interest were either introduced by site-directed mutagenesis with QuikChange XL II kit (Agilent Technologies, CA, USA) or ordered from GenScript. These nucleotide changes include: c.1139C>A, c.1139C>G, c.1144T>A, c.1144T>G, c.1169A>G, c.1146C>A, c.1144C+1146A, c.1139T+1144C, c.1144_1146delTTC and the insertion of a known wild type and mutant ESE sequence from the *ACADM* gene [17].

Transient transfection experiments

Transient transfection experiments were conducted with Fugene 6 Transfection reagent (Roche Applied Biosciences) as described [17]. Chang or COS-1 cells were seeded at $2x10^5$ (resp. $3x10^5$) cells per 35 mm well and transfected with 0.8 µg of minigene construct DNA. Co-transfections with the vectors for SF2/ASF, SRp40 (generous gifts from Adrian Krainer, Cold Spring Harbor, NY), hnRNPH (generous gift from Mark McNally, University of Wisconsin) and hnRNPA1 (generous gift from Benoit Chabot, University of Sherbrooke, Canada) were performed as described [17]. After 48 hours, cells were harvested in 300 µl RLT buffer and either stored at -80°C for later processing or RNA extraction was continued according to the manufacturer's protocol of Qiagen RNA blood mini kit.

Analysis of RNA processing

Analyses of illegitimate *PAH* transcripts from patient lymphoblasts were performed according to previously described methods [18]. After harvesting, total RNA was extracted from patient lymphoblast cell lines using Qiagen RNA blood mini kit. One microgram of

isolated RNA was reverse-transcribed with iScript™ cDNA Synthesis kit (BioRad, CA, USA) containing a mix of Oligo (dT) and random hexamer primers. The cDNA from patient lymphoblasts covering exons 9 - 13 was PCR-amplified using primers PAHX9fwd (5'-TGGCCTTGCCTCTCTGGGTGC-3') and PAHDrev (5'-GACCACATTCTGTCCATGGCTTTA-3').

Amplification of *PAH* from the minigenes was performed with a minigene-specific primer pair to exclude detection of endogenous *PAH*: forward primer 11s2 (5'-GGTAACGGAGCCAACATGGTTTTACTG-3') and reverse 11as (5'-AGACTCGAGGGTAGTCTATTATCTGTT-3'). The amplification products were analyzed by 1% agarose gel electrophoresis. PCR products were gel extracted, purified, and sequenced using the BigDye Terminator Cycle sequencing v1.1 (Applied Biosystems) on an ABI Prism 3130xL Sequencer.

RNA oligonucleotide affinity purification

The affinity purification of RNA binding proteins utilized 3'-biotin-labeled RNA oligonucleotides as described, (DNA Technology Denmark) [15]. For each purification 100 pmol of RNA oligonucleotide was coupled to 100µl of streptavidin-coupled magnetic beads (Invitrogen) and incubated with HeLa nuclear extract (Cilbiotech S.A., Belgium) [17]. After washing, bound proteins were investigated by western blotting using a monoclonal mouse antibody SF2/ASF (AK96 from Zymed Laboratories (Invitrogen)), SRp40, SRp20, hnRNPH or hnRNPA1 (sc-33418, sc-13510, sc-10042 and sc-10029, Santa Cruz Biotechnology, Santa Cruz, CA).

Expression of PAH proteins and activity assay

PAH activities were determined using a novel mass spectrometry method for quantification of Phe and Tyr in cell lysates [19]. Mutations in the human *PAH* cDNA sequence in pCMV-FLAG-PAH were introduced by site-directed mutagenesis using QuikChange XL kit from Agilent Technologies (Santa Clara, CA, USA) and confirmed by DNA sequence analysis. Expression plasmids were transfected into COS-1 cells using Fugene 6 (Roche Applied Biosciences) and harvested after 48h.

Cell lysates were prepared and enzyme activity determined using previously described methods. The amount of Tyr produced was determined by LC ESI-MSMS.

Protein concentrations of all sample types were determined using Pyrogallol Red protein dye binding assay [20]. Specific PAH activities are expressed in mU/mg total protein, with mU equal to nmol Tyr produced per min.

Results

PAH exon 11 is flanked by a weak 3' splice site

We initially assessed the strength of all splice sites in the *PAH* gene to identify exons that are weakly defined and thus vulnerable to mutations affecting splicing regulatory elements [12]. Table 4.2A displays all splice sites of the 13 exons of the *PAH* gene and the calculated strengths using the maximum entropy (MaxEntScan http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) which showed exon 11 is vulnerable with a weak 3' splice site.

Table 4.2A: PAH Splice Site Strength

Intron	5′ splice site	ME score	3′ splice site	ME score
1	cagGTgagc	9.60	aaatgcatcttatcctgtAGgaa	8.00
2	gagGTcagt	7.70	ctccccattctctcttctAGgag	11.78
3	cagGTaaga	10.77	ttgccttctctgtgtttcAGtgc	11.04
4	cctGTgagt	7.21	aggtgtctcttttctcctAGggt	8.81
5	ccaGTgagt	8.28	attttgtgcctgtattctAGtgg	7.29
6	agaGTaagt	9.35	ctttcttcttttcatcccAGctt	7.65
7	accGTgagt	9.40	ctgtgctttctgtctttcAGtga	11.57
8	cagGTaagg	11.08	ctattttcccccaattacAGgaa	9.51
9	acaGTaagt	9.49	agattgactttccattccAGatt	7.71
10	cagGTatga	9.46	ttttcacttggggcctacAGtac	3.16
11	aagGTgagg	9.16	gcctgtggttttggtcttAGgaa	8.92
12	acaGTaagt	9.49	gatggtgtttttctttgtAGgtg	10.48

5′ splice site 9mer, 3′ splice site 23mer, maximum entropy scores from http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html.

The c.1066-3C>T mutation, which is located in the 3' splice site has previously been demonstrated to result in exon 11 skipping [18], although this change only modestly weakens the score (3.16 to 2.11, Table 4.2B) and the mutant splice site retains within the splice site consensus. Moreover, c.1197A>T, an exonic mutation that only decreases the MaxEnt score of the 5' splice site from 9.16 to 7.65 has also been reported to cause exon 11 skipping [14].

Table 4.2B: Maximum entropy score variation for 3'splice site of PAH exon 11 upon c.1066-3C>T mutation

Intron	Mutation	3′ splice site	ME score
10	Wild-type (c.1066-3C)	ttttcacttggggcctacAGtac	3.16
10	c.1066-3T	ttttcacttggggcctatAGtac	2.11
10	c.1066-3T opt.	ttttcacttttttcctatAGtac	8.52
10	Wild type opt.	ttttcacttttttcctacAGtac	9.58

Together these data indicated that exon 11 is weakly defined and is likely to be dependent on exonic splicing enhancer sequences. We therefore hypothesized that mutations in exon 11 might disrupt the fine balance between exonic splicing enhancers (ESE) and exonic splicing silencers (ESS) and thus result in exon 11 skipping. We decided to investigate some of the missense mutations reported to be BH_4 responsive.

Aberrant PAH splicing in patient lymphoblast cell lines

PAH pre-mRNA processing was investigated using patient derived lymphoblast cell lines. RT-PCR was used to amplify a fragment from *PAH* exon 9 to the end of the coding region. In patients 1, 2 and 3 the expected 463 bp fragment was observed along with a 329 bp fragment (Figure 4.1A). This alternative product is most pronounced in patient 1 who is homozygous for the c.1066-3C>T mutation. DNA sequence analysis of the purified 329 bp band showed that exon 11 was missing. This analysis indicates that the c.1066-3C>T and c.1144T>C mutant alleles cause exon 11 skipping in patient cells.

Skipping of exon 11 results in deletion of 134 bp from the *PAH* mRNA leading to a shifted reading frame and replacement of the 97 C-terminal codons with 21 missense codons followed by three in frame premature stop codons in exon 12, which is the penultimate exon of the *PAH* gene. The first premature stop codon in exon 11 skipped *PAH* mRNA is located 53 nucleotides upstream of the last exon-exon junction. This corresponds to the required minimal distance (50-55 nt) upstream of the last exon, which typically triggers degradation of the premature stop codon containing mRNA by the Nonsense Mediated Decay (NMD) system [21]. There are, however, examples where NMD is triggered by premature stop codons located even closer to the last exon-exon junction [21]. To determine whether NMD is degrading the aberrantly spliced *PAH* mRNA, we treated patient cells overnight with cycloheximide (CHX), to block NMD. Assessment of *PAH* mRNA following CHX treatment showed a dramatic increase in the 329 bp cDNA product lacking exon 11 which demonstrates that the aberrantly spliced *PAH* mRNA is degraded by NMD (Figure 4.1B). The presence of the full length product confirms that the c.1139C>T mutant allele does not lead to exon 11 skipping.

The minor DNA species migrating close to the wild type transcript is a heteroduplex formed between cDNA strands of the wild type and exon 11 deleted mutant. This was confirmed by sequencing.

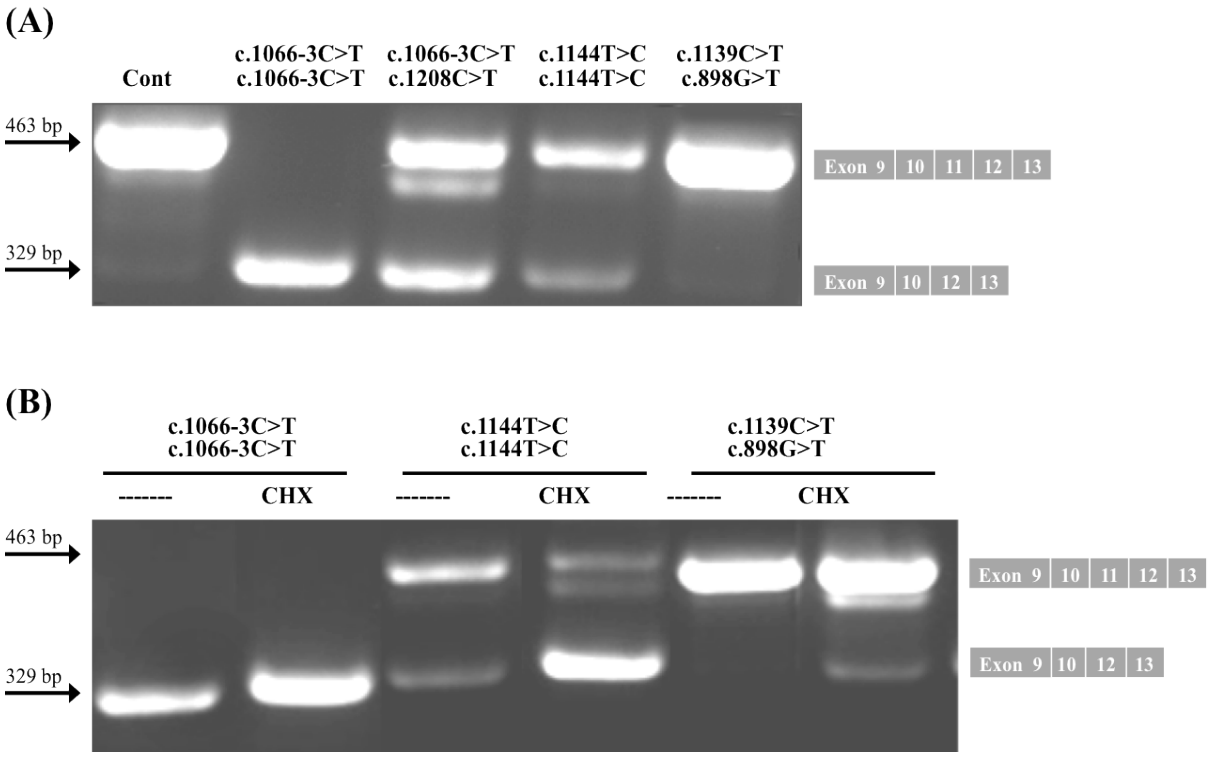


Figure 4.1: *PAH* pre-mRNA splicing analysis in patient lymphoblast cells. Cells from control and patients were analyzed with and without blockage of NMD by CHX treatment. Genotypes: Patient 1: c.1066-3C>T/c.1066-3C>T, Patient 2: c.1066-3C>T/c.1208C>T, Patient 3: c.1144T>C/c.1144T>C, Patient 4: c.1139C>T/c.898G>T. (A) Comparison of *PAH* mRNA transcript from lymphoblast patient cells by amplification of exons 9 - 13. (B) Comparison of *PAH* transcript from lymphoblast patient cells treated with 10 µg cycloheximide (CHX). The cells were passaged the day before harvesting and supplemented with CHX.

Minigene analysis confirms results from patients' lymphoblasts

To further elucidate the molecular mechanism of aberrant *PAH* exon 11 splicing and to enable testing of exon 11 mutations where cell lines are not available, a *PAH* minigene was constructed. The minigene harbors exons 10, 11 and 12, with intron 10 and a shortened intron 11 (Figure 4.2A). We tested the exon 11 mutations c.1066-3C>T, c.1139C>T, c.1144T>C, in addition to c.1197A>T that was previously reported to cause exon 11 skipping [22, 14] and c.1169A>G which is a prevalent BH_4 responsive allele in the Turkish population [10]. Analysis of COS-1 cells transfected with the minigenes shows that the mutations c.1197A>T, c.1144T>C and c.1066-3C>T lead to exon 11 skipping, whereas the c.1169A>G mutation is neutral and the c.1139C>T mutation seems to have a slightly positive effect on exon 11 inclusion (Figure 4.2B). These results are consistent with our analysis of the patient cells and underlines that the splicing of *PAH* transcripts from our minigene mimics that of the endogenous *PAH* gene. In addition, the minigene analysis confirms that the c.1197A>T, c.1144T>C and c.1066-3C>T mutations compromise splicing and that the observed missplicing in patient cells does not result from a linked mutation located outside the

sequenced region of the gene or from the other mutant allele present in the compound heterozygous patients. The minigene analysis also indicates that splicing of wild type *PAH* exon 11 results in small amounts of exon 11 skipping, consistent with the fact that it is weakly defined and dependent on ESE's [12].

As PAH is primarily expressed in the liver, we also transfected Chang cells with PAH minigene constructs. A higher degree of missplicing of the c.1144T>C minigene (Figure 4.2C) was observed, but the degree of missplicing was inconsistent between separate transfection experiments. As such, COS-1 cells were used in subsequent experiments. The results from Chang cells indicated that the c.1144T>C mutation may result in different degrees of missplicing varying from complete skipping to the same degree of skipping as observed in the COS-1 cells.

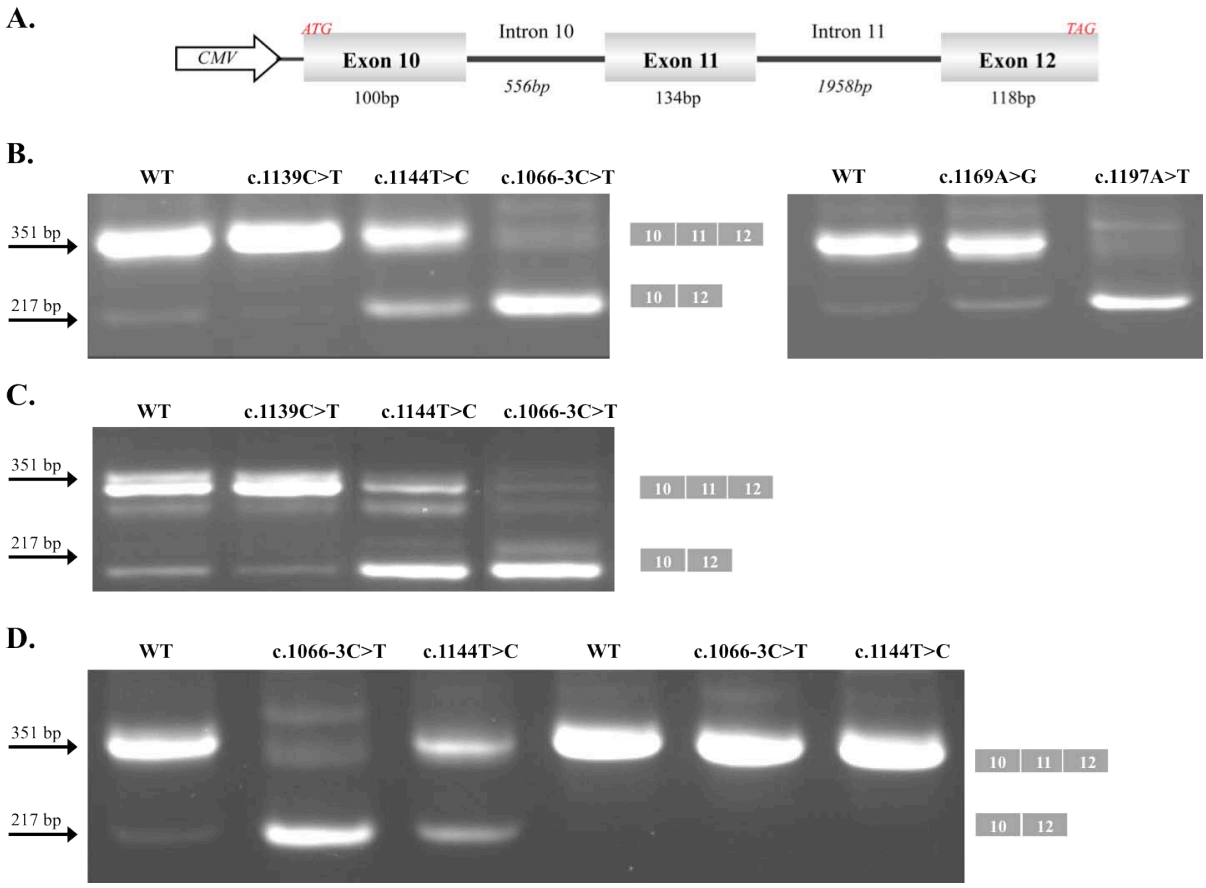


Figure 4.2: *PAH* pre-mRNA splicing in COS-1 and Chang cells transfected with *PAH* minigenes. (A) Schematic description of the *PAH* minigene harboring exons 10 to 12. Start and stop codons were added to complete the reading frame. (B) Analysis of COS-1 cells transfected with minigenes harboring WT, c.1139C>T, c.1144T>C, c.1066-3C>T, c.1169A>G and c.1197A>T mutations. (C) Analysis of two different transfections in Chang liver cells with minigenes harboring WT, c.1139C>T, c.1144T>C and c.1066-3C>T mutations. (D) Analysis of COS-1 cells transfected with minigenes harboring WT, c.1144T>C and c.1066-3C>T mutations with and without an optimized 3' splice site. Amplification of *PAH* exons 10 – 12 was done with minigene-specific primers. Transfection experiments were performed as at least two independent transfections.

This also illustrates that the degree of missplicing may vary dependent on the cell type. Heteroduplex formation of wild type and exon 11 deleted mutant cDNAs was confirmed by sequencing in both cell lines.

Correction of the 3' splice site of exon 11 by mutagenesis

To investigate the contribution of the weak 3' splice site to exon skipping in PAH exon 11, the 3' splice site was optimized. The four guanosine nucleotides (c.1066-8 to c.1066-11) that interrupt the polypyrimidine tract were replaced with thymidines. This substitution increased the maximum entropy score from a weak 3.16 to a robust 9.58. In the wild type minigene construct, improved exon 11 inclusion was observed (Figure 4.2D). Moreover, when the optimized polypyrimidine tract is included in the c.1066-3C>T and c.1144T>C constructs, aberrant splicing is no longer observed. At position -3 upstream of the 3' splice site, a thymidine base is considered to match the consensus motif. *PAH* exon 11 skipping owing to the c.1066-3C>T mutation is an indication to the weakness of the splice site owing to guanosine bases at -8 to -11. Amelioration of missplicing owing to c.1144T>C with the corrected polypyrimidine tract suggests that the c.1144T>C mutation disrupts the function of an ESE, which is required for recognition of the weak 3' splice site.

The c.1144T>C mutation disrupts an ESE, which is required for inclusion of PAH exon 11.

The c.1144T>C mutation causes exon 11 skipping. To elucidate whether the mechanism underlying pre-mRNA missplicing is owing to disruption of an ESE or creation of an ESS in the minigene constructs, various proportions of the sequence surrounding position c.1144 were deleted (Figure 4.3A). Minigenes with a 9 bp deletion (c.1141 to c.1149) showed complete exon 11 skipping, whereas the 6 bp deletion (c.1144 to c.1149) has a slightly less dramatic effect, and the 3 bp deletion (c.1144 to 1146) has a minor deleterious effect on exon 11 inclusion (Figure 4.3B). This shows that an ESE element is located in this region of exon 11, but it may also suggest that this ESE is complex and may bind more than one splicing factor, since 6 bp and 9 bp had to be deleted in order to completely abolish splicing. Alternatively, the 3 bp deletion recreates an ESE sequence (see below). To further characterize this putative ESE, we analyzed the wild type and mutant sequences with the Human Splicing Finder (HSF) program (http://www.umd.be/HSF/HSF.html) and made site-specific mutations in the region. The HSF program suggested that the c.1144T>C mutation disrupts a TTCCAG(C) ESE, which could be a binding site for the splicing stimulatory factor SRp40 (*SRSF5*). Moreover, the HSF program also suggested that a TTCCAG motif, which is

a putative ESE [23] is disrupted both by the c.1144T>C mutation and the c.1146C>A mutation, but that the c.1144T>C/c.1146C>A double mutation creates a new ESE AGCTAC. These predictions were consistent with our minigene analysis (Figure 4.3B) and suggest that the TTCCAG sequence functions as an ESE, perhaps by binding of SRp40 (*SRSF5*) and/or other factors. The HSF analysis also suggested that although the 3 bp deletion removes part of this TTCCAG motif it also recreates several potential ESE sequences, explaining the lack of a dramatic effect from this mutation. Consistent with our transfection results HSF analysis suggested a deleterious impact of both the 6 bp and the 9 bp deletions.

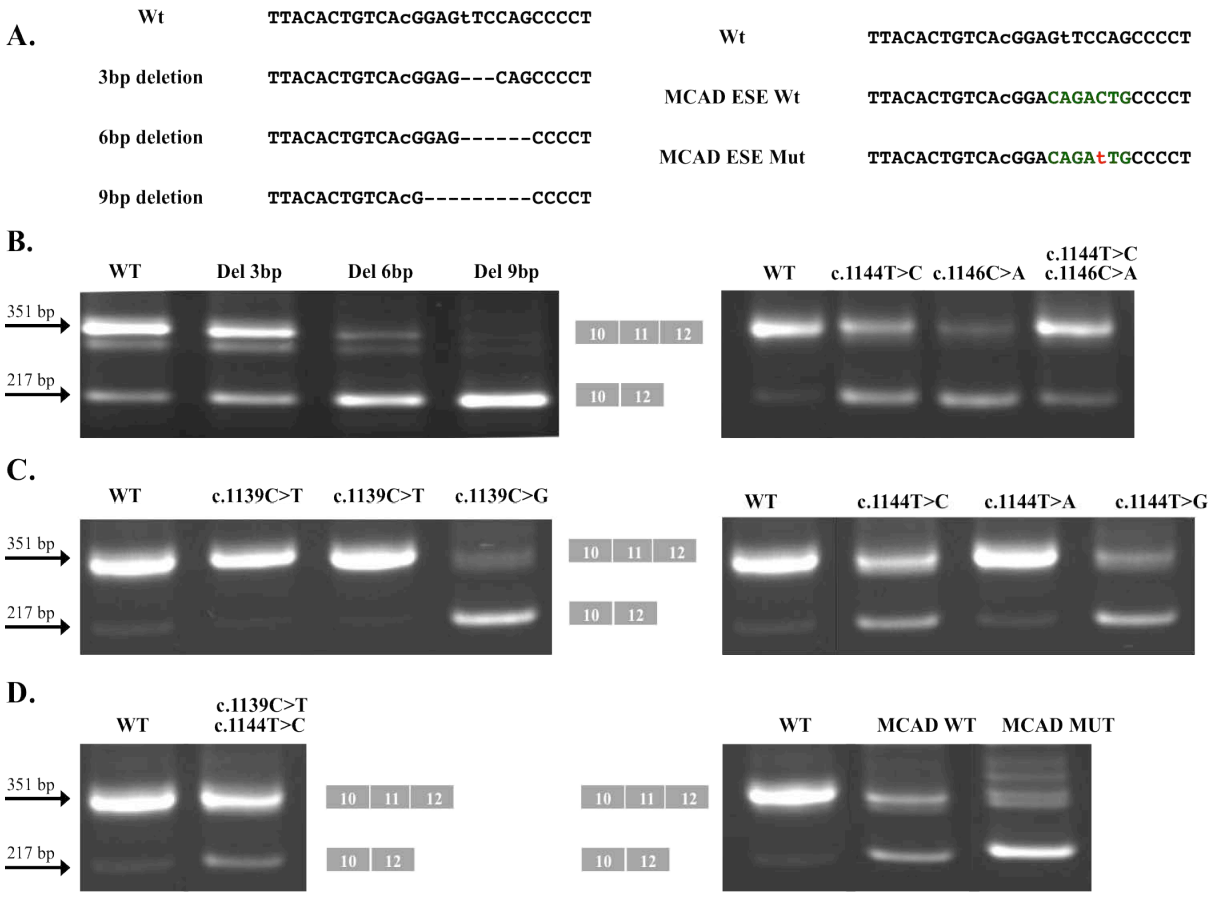


Figure 4.3: *PAH* pre-mRNA splicing in COS-1 cells transfected with *PAH* minigenes. (A) Sequences showing the precise location of the deletions and the inserted MCAD ESE. (B) Analysis of COS-1 cells transfected with minigenes harboring WT, 3 bp, 6 bp and 9 bp deletions and analysis of COS-1 cells transfected with minigenes harboring WT, c.1144T>C, c.1146C>A and c.1144T>C + c.1146C>A mutations. (C) Analysis of COS-1 cells transfected with minigenes harboring WT and all possible nucleotide variations at positions c.1139 and c.1144. (D) Analysis of COS-1 cells transfected with minigenes harboring WT, c.1139C>T, c.1144T>C, c.1139C>T+ c.1144T>C mutations and analysis of COS-1 cells transfected with minigenes harboring WT and substitutions with wild type and mutant MCAD ESE sequences. Amplification of *PAH* exons 10 – 12 was done with minigene-specific primers. Transfection experiments were performed as at least two independent transfections.

Moreover we also mutated position c.1139 and c.1144 to all possible substitutions and also created a double mutation. Interestingly, this showed that c.1139C>G and c.1144T>G both cause skipping and that introduction of the c.1139C>T mutation together with the c.1144T>C mutation compensates for the splicing defect (Figure 4.3 C, D). These data were not consistent with the HSF analysis predictions and may contradict that the ESE functions by binding of SRp40 and they suggest that the c.1139C>T mutation has a positive effect on splicing. Finally, we substituted the *PAH* ESE region with a functional ESE from the *ACADM* gene [17], which has been demonstrated to function in several other genes [17, 23]. Surprisingly, the wild type MCAD ESE could only partly substitute the *PAH* ESE sequence. As expected the mutated MCAD ESE was also non-functional in the *PAH* context (Figure 4.3 A, D). Taken together our data are consistent with a model where the c.1144T>C mutation causes exon 11 skipping by disrupting the function of an ESE, which is required for recognition of the weak 3' splice site.

Analysis of the PAH exon 11 ESE by RNA affinity purification

To identify proteins that bind the *PAH* exon 11 ESE, RNA affinity purification was performed. RNA oligonucleotides containing c.1139C>T and c.1144T>C mutant sequences were incubated in HeLa cell nuclear extracts (Figure 4.4). Western blot analysis showed strong binding of SF2/ASF (*SRSF1*) to the WT sequence, which was abolished by both the c.1139C>T and c.1144T>C mutant sequences. While factor binding was less robust for SRp20 (*SRSF3*) and SRp40 (*SRSF5*) the same pattern was observed. No difference was observed for binding of hnRNPA1. This may indicate that c.1144T>C causes exon 11 skipping by disrupting binding of an SR protein to an ESE motif that includes c.1144. However, it is not clear, if all three SR proteins bind an identical sequence motif or if they bind overlapping motifs, which are all disrupted by the c.1144T>C mutation. Alternatively one SR protein may bind the RNA while the others are associated via protein-protein interaction through their RS-domains. Moreover, it is unclear why binding of SR proteins is disrupted by c.1139C>T, as this mutation is shown to improve splicing in our minigene studies (see Figures 4.2 and 4.3). This could, however, be due to steric hindrance by binding of another protein to the new ESE created by the c.1139C>T mutation.

In support for this notion, we observed increased binding of hnRNPH to the c.1139C>T sequence and this would block binding of the SR proteins to the flanking ESE harboring position c.1144. The hnRNPH protein is, however, typically a negative regulator of splicing [15]. Further analysis is required in order to identify conclusively the involved splicing regulatory proteins that bind the exon 11 ESE.

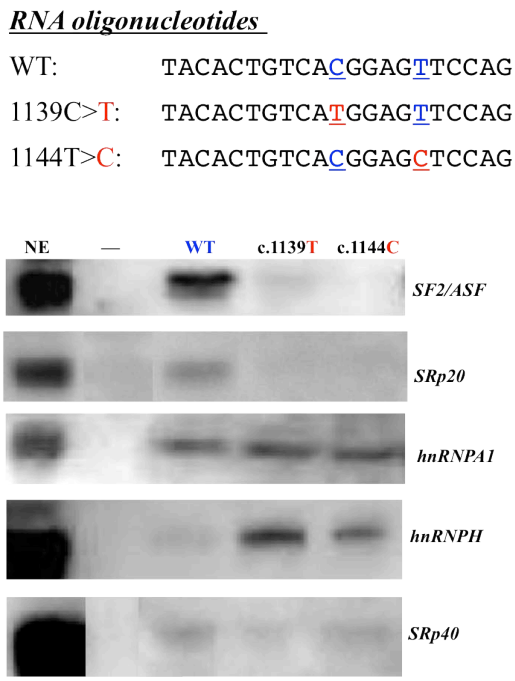


Figure 4.4: RNA oligonucleotide affinity purification. RNA oligonucleotide affinity purification of HeLa nuclear extracts using oligonucleotides with WT, c.1139C>T and c.1144T>C sequences followed by Western blot analysis.

Co-transfection with splicing factors leads to change in splicing pattern

Because the RNA affinity studies had suggested a possible role for SF2/ASF (*SRSF1*) and SRp40 (*SRSF5*) in binding to the ESE, we performed co-transfection of the *PAH* minigenes with expression plasmids for SF2/AF (*SRSF1*) or SRp40 (*SRSF5*) human proteins to see if the mutant ESE could be compensated by increasing the amounts of these SR proteins. However, instead of correcting splicing, overexpression of SF2/ASF (*SRSF1*) resulted in strong activation of a previously unknown pseudoexon (exon 11a) comprising 287bp of intron 11 and severely reduced inclusion of exon 11 (Figure 5A, B and supplementary figures S1, S2). The c.1139C>T mutant minigene had slightly less pseudoexon inclusion, consistent with the fact that this mutation results in improved splicing, possibly because it creates a new ESE, which results in stronger definition of exon 11. In line with this, pseudoexon inclusion in response to SF2/ASF (*SRSF1*) overexpression was nearly abolished when the weak 3' splice site is improved, showing that this pseudoexon activation is only possible because exon 11 is weakly defined (Figure 5C).

Similar results as above were obtained from co-transfecting the different minigenes with SRp40 (*SRSF5*) (Supplementary Figure S1). Co-transfection of hnRNPA1 and hnRNPH, two negative regulators of splicing, did not result in changes in splicing pattern or splicing efficiencies (data not shown). When patient cells are treated with cycloheximide, the new exon 11a can be amplified from patient's cDNA using an exon 11a specific primer

(Unpublished). This shows that the pseudoexon inclusion is not merely an artifact produced only from the minigenes, but that some level of pseudoexon inclusion is possible from the endogenous *PAH* gene, although the relevance of this is unclear. The pseudoexon could have deleterious effects if it is activated by mutations or by improved splicing conditions.

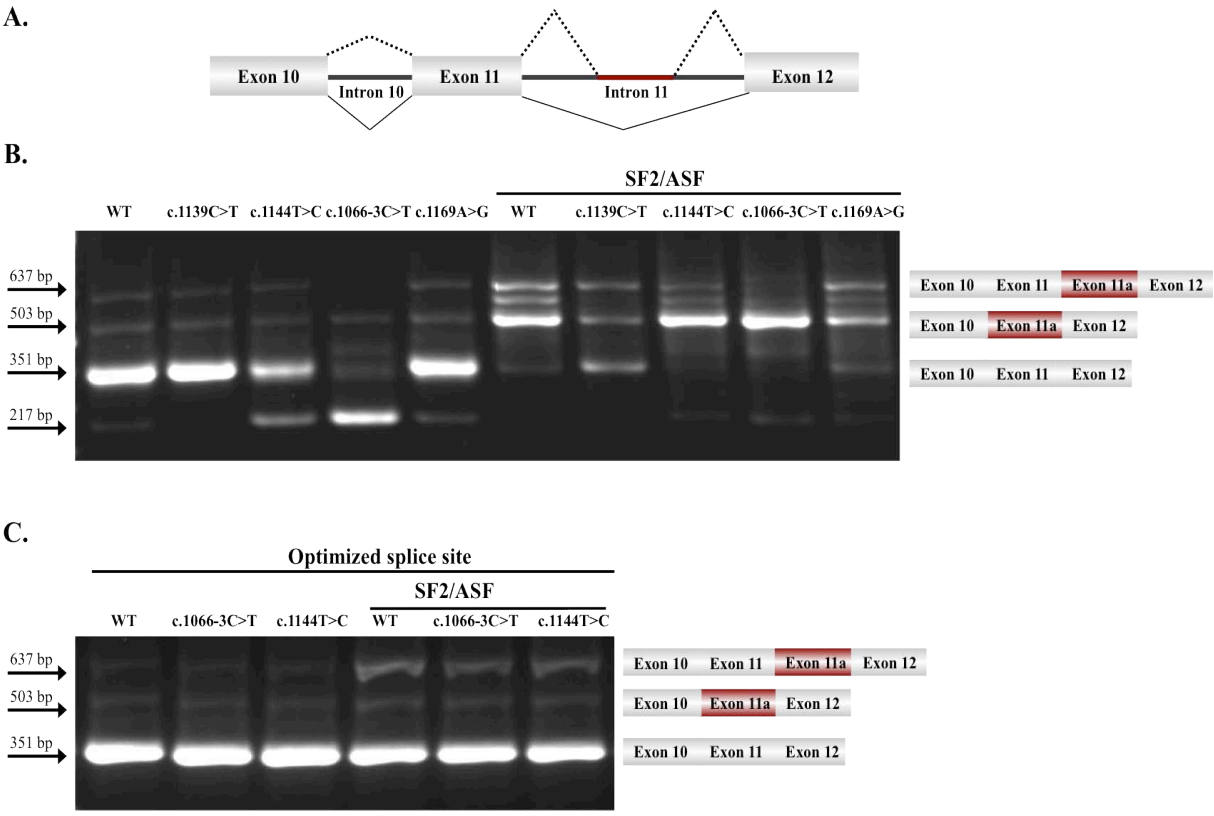


Figure 4.5: SF2/ASF overexpression in COS-1 cells transfected with *PAH* minigenes leads to pseudoexon activation. (A) Schematic overview of *PAH* splicing showing inclusion of a pseudoexon sequence from intron 11. (B) Analysis of COS-1 cells transfected with minigenes harboring WT, c.1139C>T, c.1144T>C, c.1066-3C>T and c.1169A>G mutations with and without co-transfection of a vector overexpressing SF2/ASF. (C) Analysis of COS-1 cells transfected with minigenes harboring WT, c.1144T>C and c.1066-3C>T mutations with and without and optimized 3' splice site and co-transfection of a vector overexpressing SF2/ASF. Amplification of *PAH* exons 10 – 12 was done with minigene-specific primers. Transfection experiments were performed as at least two independent transfections.

Enzyme activities of c.1139C>T and c.1144T>C are reduced compared to wild type PAH

The expression of c.1139C>T (p.T380M) and c.1144T>C (p.F382L) mutant proteins showed reduced activities compared to wild type PAH protein (Supplementary Figure S3). Residual activity for mutant p.T380M was found to be 38% of wild type activity, whereas p.F382L activity was lower with 18%. Patients with these mutations exhibit a mild phenotype, which is in accordance with residual PAH activity of 25%. The p.F382L mutant has previously been expressed in a eukaryotic expression system with a residual activity of 56%. But in this case, the amino acid change was caused by c.1146G [24].

Discussion

Correlation between genotype and disease phenotype is fundamental to inform about treatment in inherited diseases. The utility of *PAH* genotyping is increasingly relevant as efficient newborn screening is a facilitator of patients having their first clinic visit often within the first week of life. As Phe levels in early identified patients will not have reached peak concentrations, discerning disease phenotype often relies on the *PAH* genotype. The *PAH* genotype may also inform on the utility of BH_4 therapy. Furthermore *in vitro* biochemical characterization of missense mutations may provide clues as to efficacy of BH_4. However, in recent years it has become increasingly clear that a so called "splicing code" is also in operation and mutations in the coding sequence may affect pre-mRNA processing and thus overrule what can be predicted based on assumed amino acid substitutions. The splicing code is poorly defined, but it is clear that not all exons are equally subject to aberrant splicing by mutations affecting *cis*-acting splicing regulatory elements. So-called "weak exons" may often be on the verge of not being recognized, whereas other well-defined exons are easily recognized by the splicing machinery. Since the primary determinants for exon definition is the strength of their flanking splice sites, we first evaluated the splice sites of all exons of the *PAH* gene. From this analysis exon 11 clearly stood out by having the weakest 3' splice site of all exons of the *PAH* gene, and we therefore hypothesized that exon 11 could be a vulnerable exon to aberrant mRNA processing. This is further corroborated by the fact that mutations that only very modestly affected splice site strength had been reported to cause aberrant splicing of exon 11 [13,17]. Consequently, we investigated in more detail how exonic mutations may affect *PAH* exon 11 splicing.

Analysis of patient cells showed that both c.1066-3C>T and a missense mutation, c.1144T>C cause exon 11 skipping. This corroborates our hypothesis that exon 11 is vulnerable and exemplifies that simple comparison of splice site strength may help to identify vulnerable exons. Moreover, we demonstrate that NMD may lead to an underestimation of the degree of exon 11 skipping when analyzing patient derived cell lines. Similar analyses of patient cells may therefore easily overlook exon 11 skipping if NMD is not blocked prior to analysis. In order to enable investigation of patient mutations without the need of obtaining cells and to enable analysis of exon 11 splicing in more detail we established a minigene, which closely mimics the endogenous *PAH* gene. Minigenes confirmed that the c.1144T>C mutation affects splicing; moreover our testing of other artificial mutations (like c.1139C>G, c.1144T>G and c.1146C>A) shows that other exonic variants lead to exon 11 skipping. Minigene assessment demonstrated that the patient mutations c.1139C>T and c.1169A>G do

not affect splicing. Our mutagenesis of the minigene showed very clearly, that it is the weak 3' splice site that is responsible for the vulnerability of exon 11, since increasing the splice site strength by optimization of the polypyrimidine tract neutralized the effect of all the splicing mutations.

The c.1066-3C>T mutation has been first reported by Abadie et al. [18] The *PAH* amplification by RT-PCR from lymphoblast cells of their patient revealed two different transcripts, corresponding to normal spliced *PAH* and exon 11 skipped *PAH* transcripts. However, the patient was compound heterozygous for p.R261Q/c.1066-3C>T and the normal *PAH* transcript may exclusively result from p.R261Q allele. A BH_4-responsive, homozygous c.1066-3C>T patient has been reported by Desviat et al. [26]. We were not able to detect normal spliced PAH transcript in our homozygous patient for c.1066-3C>T. In addition, our patient was not responding to BH_4 in an 8 h test. To our knowledge, *PAH* gene transcript amplification from a homozygous patient was not reported before. Lymphoblasts are not the primary tissue for PAH protein expression, but we speculate that very small amounts of normal spliced *PAH* transcript could be present in liver. Faint bands observed in our transfected COS-1 and Chang cells indicate that low amounts of normally spliced *PAH* can be produced from the c.1066-3C>T. Even low amounts of *PAH* transcript would be stabilized by BH_4 and result in lowered Phe levels upon BH_4 treatment.

Because testing mutations using minigenes and/or patient cells is cumbersome there is a growing need for computer-based predictions of possible deleterious effects on splicing. The Human Splicing Finder program simultaneously analyzes wild type and mutant sequences for changes in splicing regulatory sequences and theoretical binding motifs for splicing factors. Although, such programs may provide useful hints to potential regulatory sequence motifs, which are changed by a mutation, the present study demonstrates that predictions cannot be used uncritically and that the different algorithms may produce contradictory predictions. In the present study the c.1139C>T mutation is predicted to be deleterious, but instead it seems to improve splicing, which demonstrates why functional testing is still warranted. Alternatively, HSF predictions concerning the c.1144T>C mutation and in particular identifying TTCCAG (c.1144-c.1148) as an ESE may be correct. The importance of this sequence is further demonstrated by the fact that it is conserved in species ranging from elephants to mice (Figure 4.6). Moreover, comparison with our database of other exonic splicing mutations (unpublished observation) showed that a G>T SNP in exon 4 of the *CYP2B6* gene (rs3745274) [27], which causes aberrant splicing, disrupts the same motif at a different position (TTCCAG), and is also predicted to be deleterious by HSF.

Homology: TTCCAG ESE motif is conserved in all species.

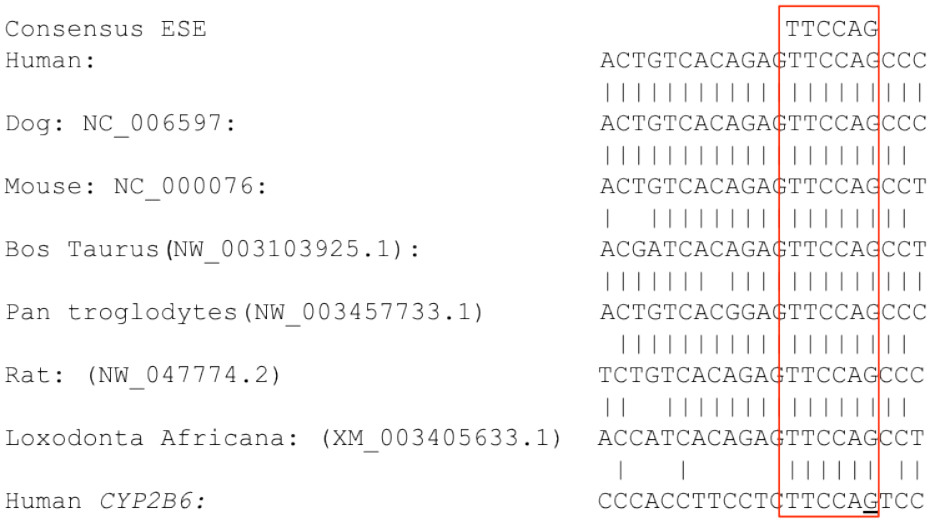


Figure 4.6: Alignment of *PAH* exon 11 sequences from different species. Alignment of sequences from different species shows that the TTCCAG (c.1144 - c.1148) sequence is conserved in different species and that mutation (underlined G) in this element causes aberrant splicing in the human *CYP2B6* gene.

This indicates that *cis*-acting motifs are general and may be functional in other genes. On the other hand, the well-characterized ESE from the *ACADM* gene [17, 24] has been demonstrated to function in other genes, failed to replace the endogenous *PAH* ESE sequence. The failure of the *ACADM* ESE shows that motifs are not completely interchangeable, but that there are exon-specific requirements, possibly reflecting the need for recruitment of different splicing regulatory proteins. Our preliminary attempts to identify the factor(s) that binds to the *PAH* ESE spanning position c.1144 were not conclusive, although they very clearly showed that there were dramatic differences in the binding of important splicing regulatory proteins to wild type and mutant *PAH* sequences.

We conclude that our study shows that exon 11 of the *PAH* gene is a vulnerable exon due to its weak 3' splice site and that this makes exon 11 inclusion dependent on an ESE spanning position c.1144. Importantly, this implies that also a number of other mutations in exon 11 are likely to affect splicing, since splicing is often determined by a fine balance between several positive and negative splicing regulatory elements distributed throughout the exon. It is therefore important to assess the effect of all mutations in exon 11 on splicing by using our established minigene, since such mutations that disrupt splicing are unlikely to facilitate response to BH_4 and if not recognized their effect on splicing may lead to inconsistent genotype-phenotype correlations.

Acknowledgments

This work was supported by grants from The Danish Medical Research Council (FSS grants no. 271-07-342 and no.11-107174) to BSA and Swiss National Science Foundation grant no. 31003A-119982/2 to NB and National Institutes of Health grant no. R44HD075156 and no. R44DK069106 to SFD. We thank Prof. D. Nadal from the Children's Hospital Zurich for the help with the establishment of lymphoblast cell lines.

References

[1] N. Blau, F.J. Van Spronsen, H.L. Levy, Phenylketonuria Lancet 376 (2010) 1417-1427.

[2] S. Kaufman, New tetrahydrobiopterin-dependent systems Annu Rev Nutr 13 (1993) 261-286.

[3] C.R. Scriver, S. Kaufman, Hyperphenylalaninemia: Phenylalanine hydroxylase deficiency, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, B. Vogelstein (Eds.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 1667-1724.

[4] C.R. Scriver, The PAH gene, phenylketonuria, and a paradigm shift Hum Mutat 28 (2007) 831-845.

[5] A.L. Pey, F. Stricher, L. Serrano, A. Martinez, Predicted effects of missense mutations on native-state stability account for phenotypic outcome in phenylketonuria, a paradigm of misfolding diseases Am J Hum Genet 81 (2007) 1006-1024.

[6] F. Pagani, F.E. Baralle, Genomic variants in exons and introns: identifying the splicing spoilers Nat Rev Genet 5 (2004) 389-396.

[7] N. Blau, Sapropterin dihydrochloride for phenylketonuria and tetrahydrobiopterin deficiency Expert Rev Endocrinol Metab 5 (2010) 483-494.

[8] A.C. Muntau, W. Röschinger, M. Habich, H. Demmelmair, B. Hoffmann, C.P. Sommerhoff, A.A. Roscher, Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria N Engl J Med 347 (2002) 2122-2132.

[9] I. Karacic, D. Meili, V. Sarnavka, C. Heintz, B. Thony, D.P. Ramadza, K. Fumic, D. Mardesic, I. Baric, N. Blau, Genotype-predicted tetrahydrobiopterin (BH4)-responsiveness and molecular genetics in Croatian patients with phenylalanine hydroxylase (PAH) deficiency Mol Genet Metab 97 (2009) 165-171.

[10] S.F. Dobrowolski, C. Heintz, T. Miller, C.R. Ellingson, C.C. Ellingson, I. Özer, G. Gökcay, T. Baykal, B. Thöny, M. Demirkol, N. Blau, Molecular genetics and impact of residual in vitro phenylalanine hydroxylase activity on tetrahydrobiopterin-responsiveness in Turkish PKU population Mol Genet Metab 10 (2011) 116-121.

[11] F.K. Trefz, D. Scheible, H. Gotz, G. Frauendienst-Egger, Significance of genotype in tetrahydrobiopterin-responsive phenylketonuria J Inherit Metab Dis 32 (2009) 22-26.

[12] B.S. Andersen, A.R. Krainer, When the genetic code is not enough – How sequence variations can affect pre-mRNA splicing and cause (complex) disease, in: L. Almasy, A. Al-Chalabi (Eds.), Genetics of Complex Human Diseases, Cold Spring Harbor Laboratory Press, New York, 2009, pp. 165-182.

[13] S. Ellingsen, P.M. Knappskog, H.G. Eiken, Phenylketonuria splice mutation (EXON6nt-96A-->g) masquerading as missense mutation (Y204C) Hum Mutat 9 (1997) 88-90.

[14] H.K. Chao, K.J. Hsiao, T.S. Su, A silent mutation induces exon skipping in the phenylalanine hydroxylase gene in phenylketonuria Hum Genet 108 (2001) 14-19.

[15] S.F. Dobrowolski, H.S. Andersen, T.K. Doktor, B.S. Andresen, The phenylalanine hydroxylase c.30C>G synonymous variation (p.G10G) creates a common exonic splicing silencer Mol Genet Metab (2010).

[16] P.M. Brickell, Immortalization of human B-lymphocytes by epstein-barr virus Methods Mol Biol 8 (1992) 213-218.

[17] K.B. Nielsen, S. Sorensen, L. Cartegni, T.J. Corydon, T.K. Doktor, L.D. Schroeder, L.S. Reinert, O. Elpeleg, A.R. Krainer, N. Gregersen, J. Kjems, B.S. Andresen, Seemingly neutral polymorphic variants may confer immunity to splicing-inactivating mutations: a synonymous SNP in exon 5 of MCAD protects from deleterious mutations in a flanking exonic splicing enhancer Am J Hum Genet 80 (2007) 416-432.

[18] V. Abadie, J. Jaruzelska, S. Lyonnet, P. Millasseau, M. Berthelon, F. Rey, A. Munnich, J. Rey, Illegitimate transcription of the phenylalanine hydroxylase gene in lymphocytes for identification of mutations in phenylketonuria Hum Mol Genet 2 (1993) 31-34.

[19] C. Heintz, H. Troxler, A. Martinez, B. Thöny, N. Blau, Quantification of phenylalanine hydroxylase activity by isotope-dilution liquid chromatography–electrospray ionization tandem mass spectrometry Mol Genet Metab (2012) doi:10.1016/j.ymgme.2011.1012.1025.

[20] N. Watanabe, S. Kamei, A. Ohkubo, M. Yamanaka, S. Ohsawa, K. Makino, K. Tokuda, Urinary protein as measured with a pyrogallol red-molybdate complex, manually and in a Hitachi 726 automated analyzer Clinical chemistry 32 (1986) 1551-1554.

[21] J. Hwang, L.E. Maquat, Nonsense-mediated mRNA decay (NMD) in animal embryogenesis: to die or not to die, that is the question Curr Opin Genet Dev 21 (2011) 422-430.

[22] J. Jaruzelska, V. Abadie, Y. d'Aubenton-Carafa, E. Brody, A. Munnich, J. Marie, In vitro splicing deficiency induced by a C to T mutation at position -3 in the intron 10 acceptor site of the phenylalanine hydroxylase gene in a patient with phenylketonuria J Biol Chem 270 (1995) 20370-20375.

[23] A. Goren, O. Ram, M. Amit, H. Keren, G. Lev-Maor, I. Vig, T. Pupko, G. Ast, Comparative analysis identifies exonic splicing regulatory sequences-The complex definition of enhancers and silencers Mol Cell 22 (2006) 769-781.

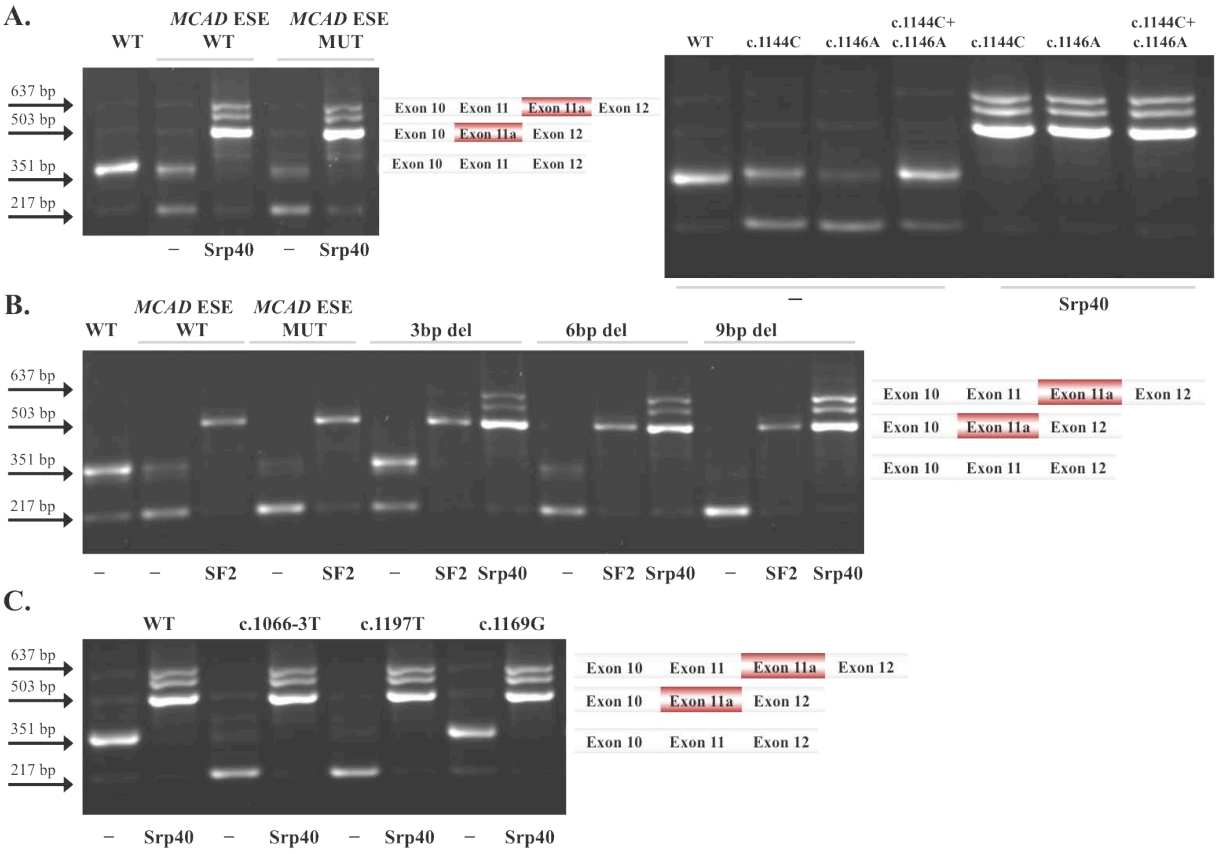
[24] I. Tournier, M. Vezain, A. Martins, F. Charbonnier, S. Baert-Desurmont, S. Olschwang, Q. Wang, M.P. Buisine, J. Soret, J. Tazi, T. Frebourg, M. Tosi, A large fraction of unclassified variants of the mismatch repair genes MLH1 and MSH2 is associated with splicing defects Hum Mutat 29 (2008) 1412-1424.

[25] A. Daniele, G. Cardillo, C. Pennino, M.T. Carbone, D. Scognamiglio, L. Esposito, A. Correra, G. Castaldo, A. Zagari, F. Salvatore, Five human phenylalanine hydroxylase proteins identified in mild hyperphenylalaninemia patients are disease-causing variants Biochim Biophys Acta 1782 (2008) 378-384.

[26] L.R. Desviat, B. Pérez, A. Bèlanger-Quintana, M. Castro, C. Aguado, A. Sánchez, M.J. García, M. Martínez-Pardo, M. Ugarte, Tetrahydrobiopterin responsiveness: results of the BH_4 loading test in 31 Spanish PKU patients and correlation with their genotype, Mol. Genet. Metab. 82 (2004) 157–162.

[27] M.H. Hofmann, J.K. Blievernicht, K. Klein, T. Saussele, E. Schaeffeler, M. Schwab, U.M. Zanger, Aberrant splicing caused by single nucleotide polymorphism c.516G>T [Q172H], a marker of CYP2B6*6, is responsible for decreased expression and activity of CYP2B6 in liver J Pharmacol Exp Ther 325 (2008) 284-292.

Supplementary Figure S1: SRp40 and SF2/ASF overexpression in COS-1 cells transfected with *PAH* minigenes also leads to pseudoexon activation.

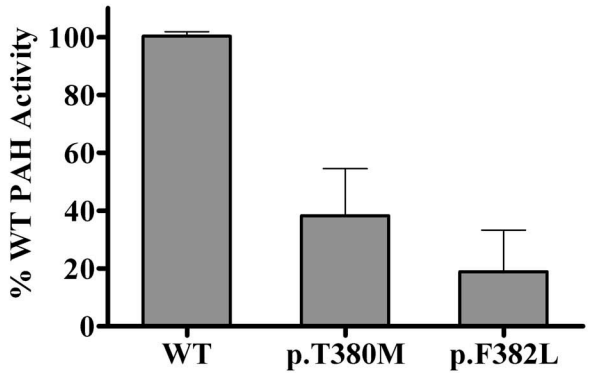


(A) Analysis of COS-1 cells transfected with minigenes harboring WT and MCAD wild type and mutant ESE inserted and WT, c.1144T>C, c.1146C>A and c.1144T>C + c.1146C>A mutations. (B) Analysis of COS cells transfected with minigenes harboring WT, MCAD ESE substitutions or deletions of 3 bp, 6 bp or 9 bp. (C) Analysis of COS-1 cells transfected with minigenes harboring WT, c.1066-3C>T, c.1197A>T or c.1169A>G mutations. All transfections were carried out with or without co-transfection of a vector overexpressing SRp40 or SF2/ASF. Amplification of *PAH* exons 10 - 12 was done with minigene-specific primers. Transfection experiments were performed as at least two independent transfections.

Supplementary Figure S2: *PAH* intron 11 pseudoexon sequence. The pseudoexon corresponds to position 65379907 - 65380192 in NT_029419.12.

*agaaattttgttctttctattttctgttttcctccag*CCCCTGGAACAATACC
TGACACAGAGTAAGCACTCAATAAATAATTGGTGAAGGCATTAATCCCAGAGGC
AGTTCAGCAAGAGGCTGATGGAGTGGTTCTGGGACTCTGAGACTGTTGGCCCTC
TGAGTGCACAGTTCCTGCACCCAAGCAGGAGACAGACTGGGCAGAGGGTCCACC
TGGACATGAATATCCTAATACTCACTGTATGTTACCACTTAAAAAAATCTTATC
AGTGATGGATGACAGGCATGGAACATACAGGCCTTTGGGAGTCTATGACACTGG
gtatgttagctttcaaccctatctatggcctctgagaggtcagaggaatgg

Supplementary Figure S3: PAH activities of p.T380M (c.1139C>T) and p.F382L (c.1144T>C) transiently transfected into COS-1 cells.



Mutations were introduced into intact *PAH* cDNA by site-directed mutagenesis in pCMV FLAG PAH overexpression plasmid. PAH activities are normalized for transfection efficiency by co-transfection with β-galactosidase expression vector.

Chapter 5

Co-Expression of *PAH* Mutant Alleles in Eukaryotic Cells

Biochemical Characterization and Genotype-Phenotype Correlations in BH_4-responsive PKU

Preliminary, unpublished data.

Abstract

The genotype-based classification of tetrahydrobiopterin (BH_4) responsiveness and the importance of residual PAH activity have been documented in a cohort of Croatian and Turkish PKU patients. It has been shown that single allele mutations are not reliable for the selection of potential PKU candidates for pharmacological therapy with BH_4. On the basis of these results, several mutations were characterized on protein level to elucidate BH_4-response and explain inconsistent genotype/phenotype correlations. Our goal is to document and catalog all genotype-phenotype data for easier prediction of patient's outcome. In the current project, mutations were characterized by transient expression and co-expression in eukaryotic cells and correlated with patients' phenotype and available BH_4 loading test data. With the co-expression system we aimed to elucidate interallelic interactions and mutation dominance effects in previously identified genotypes combined with *in vitro* BH_4 responsiveness data. A new highly sensitive and specific PAH assay method by LC-ESI-MSMS was used for *in vitro* PAH activity measurement. Molecular, *in vitro* characterization of genotypes, i.e. the analysis of compound heterozygote mutations by co-expression, together with comparison to thoroughly cataloged patient data will provide a sophisticated tool for the true BH_4 responsiveness diagnosis in new PKU patients.

Introduction

The investigation of the severity and impact of a mutation in a corresponding metabolic phenotype is one aim of *in vitro* expression of PAH mutations. The pathogenic effects of about 100 mutant PAH proteins have been investigated in detailed biochemical studies for a better understanding of relationships between mutant biochemistry and PKU disease presentation. Protein misfolding with increased aggregation and destabilized native conformation is a main cause of PKU and many of the *PAH* missense mutations, distributed all over the gene, influence folding and stability of the resulting enzyme. Thus, PKU disorder was found to be a good candidate for treatment by pharmacological chaperones and the natural cofactor BH_4 emerged as first FDA- and EMEA-registered therapeutic agent for some PKU patients.

The potential of BH_4 reponsiveness among PKU patients with mild to moderate phenotypes is high and thus, the *in vitro* mechanisms and testing of BH_4 responsiveness were assessed for many mutations (1, 2, 3, 4). Some mutations were associated with a response to BH_4, but for many an unclear relation was reported (5, 6). PAH is naturally active as a homotetramer and the association of different monomers to a heterotetramer highly vary depending on the type of mutation and its impact on the 3D structure. PAH deficiency therefore most often results from complex interactions of mutant alleles or rapid intracellular dissociation of mutant enzyme subunits, making genotype-phenotype correlations, based on knowledge about individual mutations, challenging (7). Around 75% of PKU patients exhibit a compound heterozygote genotype, often including a mild mutation. These patients are main targets for BH_4-loading tests, as they generally present with a significant residual PAH activity and PAH protein that can be potentially stabilized by BH_4. Still, only a few studies have correlated the genotype with an effect of BH_4 treatment and highlighted the importance of full genotype information (6, 8, 9). Although, it is now known that the genotype determines responsiveness, little is known on how a single mutation in the compound heterozygous situation affects the structure, stability, and kinetics of the heterotetrameric mutant PAH. The interaction of two mutated subunits may exhibit a different response to BH_4 and different molecular behavior as the single homozygous mutation alone. Therefore, the characterization of subunit interactions is important as it leads to better understanding and prediction of the patient's phenotype and BH_4 responsiveness.

Interallelic complementation describes the interaction between two mutant alleles at a given locus to form a multimeric protein. A particular combination of two alleles may

produce a more (negative complementation) or less (positive complementation) severe phenotype than expected from the homoallelic counterpart (10). It is possible, that complementation leads to correction of misfolding of one of the monomers (11). This phenomenon of positive or negative complementation contributes to the phenotypic severity in PKU and may explain for example the absence of BH_4-response in patients carrying two responsive alleles (12). Several experimental setups have been reported and evaluated for the study of interactions between two PAH subunits. Two-hybrid analysis allows the measurement of protein-protein interactions occurring in living cells. PAH monomers were expressed as fusion proteins and results showed that misfolded mutant PAH subunits interact to some extent and that heteromeric enzymes are formed leading to interallelic effects (13, 14).

The co-expression of two distinct mutants in bacteria using a dual vector approach or a bicistronic expression system requires the purification of the heterotetrameric proteins (12, 15). In these systems higher amounts of protein are produced that allow more detailed analysis on stability and kinetics of the heterotetrameric PAH forms in terms of residual activity, stability and affinity for BH_4. The existence of negative interallelic complementation has been demonstrated for a few allele combinations (15). However, the prokaryotic expression system is limited in testing the effect of BH_4 concentrations when the protein assembles *in vivo*. The assessment of BH_4-treatment and possible effects of responsiveness induced by only one of the mutant alleles is not feasible in such a bacterial co-expression system.

In this chapter, residual PAH activities are studied upon transient co-expression of two distinct PAH alleles in a mammalian system. The mammalian cell system provides a close approximation to the *in vivo* milieu for protein expression. PAH p.R261Q mutant is frequently reported with different phenotypes and inconsistent response to BH_4 treatment (see also Table 1.7 in Chapter 1) (16), although the mutant shows substantial residual activity (43% of the wild type, Chapter 3). In a single expression study, p.R261Q protein was reported with reduced binding affinity for Phe and no increase in half-life by addition of BH_4. Also, no activation and increase in specific affinity was found upon pre-incubation with Phe. The affinity for the cofactor was similar than in the wild type protein. (2, 3, 4). The p.R261Q allele was selected for expression in combination with several other alleles reported with inconsistent BH_4 responsiveness and the impact on residual PAH activity and BH_4 response was investigated. A dual eukaryotic vector system was used with the two mutant PAH proteins N-terminally fused to different epitope tags for discernible allele expression. The

expression combinations were chosen according to genotype data in the BIOPKU database (www.biopku.org), where identical genotypes are sometimes listed with diverging reports of BH_4 responsiveness and with significant residual activity of the second allele. The response to BH_4 of a given allele combination was analyzed by supplementation of the cell culture medium with BH_4 or sepiapterin. Sepiapterin can be reduced to BH_4 in the cell by dihydrofolate reductase (DHFR) (17). However, the response to BH_4 does not only depend on the genotype and mechanisms are probably multifactorial (6). Genetic environment and differences in BH_4 absorption or metabolism cannot be controlled, but it has been suggested by Pey et al. (18) and Staudigl et al. (19) that residual PAH activity and cofactor response depend also on the Phe level. Hence, residual activities and BH_4-response of co-expressed PAH mutants were analyzed under normal and high Phe culturing conditions.

The transient co-expression of two distinct PAH mutations in cell culture mimics most closely the patient situation and may allow testing the effect of various BH_4 and Phe levels for better prediction of BH_4 responsiveness.

Materials and Methods

Construction of mammalian expression vectors and site-directed mutagenesis

The expression plasmid pCMV-FLAG-PAH wild type (Promoter-N-Fusion-PAH) was received as courtesy gift from L. R. Desviat (20). *PAH* wild type cDNA had been cloned into pFLAG-CMV-2 vector (Sigma) using *SalI* and *NotI* restriction sites after amplification of cDNA from pMAL-PAH (21) (human *PAH* GenBank reference NM_000277.1). The pFLAG-CMV-2 vector is a high-copy plasmid with CMV promoter, leading to high levels of expression in mammalian cells. Mutations in the human *PAH* cDNA sequence were introduced by site-directed mutagenesis using QuikChange XL kit from Agilent Technologies (Santa Clara, CA, USA). The online primer design program offered by Agilent was used to design mutagenic primers (http://www.genomics.agilent.com/HomePage.aspx). The pCMV-FLAG-PAH wild type plasmid was the template for replacement of FLAG tag by c-Myc tag in two sequential rounds of mutagenesis to generate pCMV-Myc-PAH wild type (Plasmid maps in Figure 5.1). Lyophilized primer stocks were ordered from Microsynth AG (Switzerland).

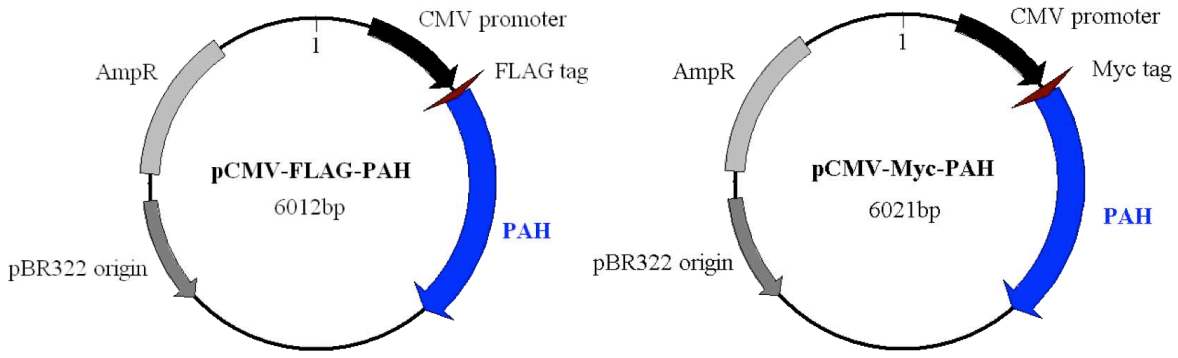


Figure 5.1: Plasmid maps of human PAH expression vectors.

The following three primers were used to sequence complete PAH cDNA:

Table 5.1: Primers used for sequencing of hPAH cDNA

Name	Sequence
pCMV-hPAH:	5'-AATGTCGTAACAACTCCGCCCCATTGACGC-3'
hPAH_2:	5'-TCTCAGCTATGGAGCGGAAC-3'
hPAH_3:	5'-CATGTATACCCCCGAACCTG-3'

Correct insertion of mutations was confirmed by sequencing analysis using BigDye Terminator Cycle sequencing v1.1 (Applied Biosystems) on an ABI Prism 3130xl Sequencer.

The following mutation combinations were chosen for transient expression in COS-1 cells:

Table 5.2: Mutation combinations planned for co-expression in COS-1 cells with residual PAH activity of second allele.

hPAH allele 1	hPAH allele 2	Residual activity allele 2 (% Wt)	Predicted residual activity (% Wt)
p.R261Q	p.L48S	39	41
p.R261Q	p.I65T	33*	38
p.R261Q	p.R408W	2*	22.5
p.R261Q	p.R408Q	55	49
p.R261Q	p.E280K	1-3	23
p.R261Q	p.R158Q	5*	24
p.R261Q	p.E390G	54*	48.5
p.R261Q	p.Y414C	57	48

All mutagenic primers are listed in the supplementary table. *-marked residual activities were determined as described in Chapter 3. The others were compiled from PAHdb Knowledgebase (22). The predicted residual activity was calculated as follows: (43% for allele p.R261Q + % allele 2)/2.

Comparison and Optimization of Mammalian Cell Transfection Methods

All cell lines (HepG2, HuH-7, Hep3B, COS-1) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich, USA), supplemented with 10% fetal calf serum, 4 mM L-Glutamine and 1% Penicillin/Streptomycin at 37 °C under 5% CO_2. One day prior to transfection a confluent 10-cm HuH-7 or COS-1 dish was split into two 6-well plates, which resulted in 50% cell confluency on the day of transfection. For a transfection experiment in 10-cm culture dishes, confluent HuH-7 or COS-1 dishes were split in a 1:6 ratio one day before transfection. Hep3B cells were transfected only in 10-cm dishes with a split ration of 1:3 on the day before transfection. The following transfection reagents were compared in 6-well cell culture plates for transfection efficiency and scaled up to 10-cm cell culture dishes.

Manufacturer's guidelines recommending plasmid DNA amounts and transfection reagent volumes were followed for Jetpei, Fugene 6 and Lipofectamine reagents. Pei200 transfections were performed similar to the Jetpei protocol using N/P ratios (measure of ionic balance of complexes) (25, 26). DEAE/Dextran transfections were performed according to (27).

Table 5.3: Transfection reagents tested in HuH-7, Hep3B and COS-1 cell lines.

Chemical transfection method	Properties
Jetpei (Polyplus, France)	Linear polyethylenimine (Pei), 25kDa MW
Pei200 (23, 24)	Linear deacylated Pei, 87kDa MW
Fugene 6 (Roche Applied Sciences, Germany)	Multi-component nonliposomal reagent
DEAE/Dextran (Sigma Aldrich, USA)	Cationic polymer
Lipofectamine 2000/LTX (Invitrogen Life Technologies, USA)	Cationic and neutral lipids, forming liposomes (LTX = advanced formulation)

MW = molecular weight

Efficiencies were assessed by transfecting pGeneGrip-GFP plasmid (Genlantis, USA) with CMV promoter using fluorescence microscopy. Staining of the cell nuclei was achieved with Hoechst dye 33342. Different ratios for transfection reagent/DNA mixtures were tested. Variations in transfection conditions included change of serum concentration (0 or 10%) or the use of Opti-MEM reduced serum medium (Invitrogen) for preparation of transfection complexes and/or incubation of cells.

Expression of PAH mutants in COS-1 cells

One day prior to transfection, COS-1 cells were split in a 1:5 or 1:6 ratio (corresponding to $\sim 2 \times 10^5$ cells/mL) in 10-cm dishes. Transfection experiments were performed using Fugene 6 (Roche Applied Sciences) according to manufacturer's recommendations. Hereby, 13 µg of the pCMV-FLAG-PAH plasmid (either wild type or mutant) were co-transfected with 2 µg of pSV-βgal reporter plasmid (Promega, Madison, USA) using 30 µL of Fugene 6 reagent in serum-free medium. Transfection experiments always included expression of wild type PAH, several mutant PAH constructs and the comparison to non-transfected COS-1 cells. In addition, one COS-1 10-cm cell plate was transfected with 15 µg pSV-βgal vector and stained using in-situ β-galactosidase staining kit (Agilent Technologies, USA) after 48 h. PAH-transfected cells were harvested after 48 h for immediate determination of PAH activity or flash-frozen in liquid N_2 for storage at -80°C.

Transfection efficiency in co-transfected cell pellets was verified by determining β-galactosidase activity in 5-10 µL lysate in PAH assay cell lysis buffer (1× PBS pH 7.4, 0.25 M sucrose, complete protease inhibitors cocktail (Roche)) using in-situ β-galactosidase enzyme assay system (Promega, Madison, USA). PAH activities of wild type and mutants were normalized using the absorbance results at 420 nm of *o*-nitrophenol (yellow color

development at 37°C with a linear range between OD_{420} 0.1 and 0.9) as measure of transfection efficiency.

PAH activity assay

The PAH activity assay was performed according to (28, 29). In brief, cell pellets were lysed after harvest by 6 freeze-thaw cycles in lysis buffer (2 M KH_2PO_4/K_2HPO_4, 1.5 M KCl, protease inhibitors). For every PAH assay, cell homogenate containing 50 - 100 µg of total protein were used. The appropriate amount of homogenate was adjusted to a final volume of 77.5 µL with H_2O, followed by adding 22.5 µL of master mix containing 0.6 mM Phe, 3.6 U of catalase (Sigma), and 0.15 M KCl in a 0.2 M potassium phosphate buffer, pH 6.8. After pre-incubation at room temperature for 5 min, the reaction was started by adding 2 µL of 0.1 M dithiothreitol and 2 µL of 4.5 mM 6-methyltetrahydropterin (Schircks Laboratories, Switzerland) to the samples and incubated for 60 min at 25 rech°C. The reaction was stopped by incubation for 5 min in a 96°C heating block and centrifuged for 5 min at 13'000 x g. The blank samples without PAH enzyme were adjusted as well with the appropriate amount of cell homogenate to a final volume of 104 µL with H_2O and incubated for 5 min in a 96°C heating block.

The supernatant was filtrated in a centrifugal filter device (Amicon Ultra-0.5, UFC501096, Millipore, USA) and centrifuged at 5'000 × g for 15 min. Phe and Tyr were quantified with a standard amino acid analyzer (Biochrom 20 Plus, Amersham Biosciences).

Treatment of cells with BH_4, sepiapterin, and phenylalanine

BH_4 dihydrochloride (no. 11.212) and sepiapterin (no. 11.225) were obtained from Schircks Laboratories (Jona, Switzerland). BH_4 solution was prepared in 5 mM HCl and 50 mM DTT for increased stability, as BH_4 solution is sensitive to oxygen and basic pH. In cell culture, BH_4 was further stabilized with the addition of ascorbic acid (Vitamin C, Fluka, Switzerland) solution in a 1:1 weight ratio (30). BH_4 was added to a final concentration of 75 µM to the cell culture medium five hours after addition of the DNA/Fugene 6 mixture. Cell culture medium with BH_4 was renewed after 24 hours.

Sepiapterin solution was prepared in degased double-distilled H_2O (ddH_2O) in the dark and stored under N_2 atmosphere at -80°C in single aliquots to ensure stability. The sepiapterin solution was diluted to 75 µM in the cell culture medium, five hours after transfection.

Phe was added at a concentration of 1200 µM to the cell culture medium, three hours after transfection. Phe solution was prepared in ddH_2O and stored at -20°C.

Oxidation of cell lysates and culture medium for pterin measurement

Because BH_4 is unstable and prone to oxidation, especially at alkaline conditions, it is difficult to directly determine BH_4 concentrations analytically. This problem is circumvented by differential oxidation of the biological sample (31, 32). One hundred µL of cell medium from the medium bottle supplemented with BH_4/Vitamin C or from the cell culture plate 24 hours after addition of BH_4/Vitamin C, were added to 150 µL of 0.2 M HCl and oxidized for one hour in the dark with the addition of 1% oxidation solution (1% iodine in H_2O and 2% KI). After the addition of a 2% ascorbic acid solution, the samples were measured by reversed-phase HPLC and fluorimetric detection.

We measured biopterin and neopterin concentration in the cell lysates to confirm that sepiapterin is metabolized. The cells were lysed using PAH assay protocol (Chapter 3) and lysates were oxidized according to the protocol described by Elzaouk et al. (33). At acidic conditions, iodine solution (0.5%) was therefore added to the lysates, followed by incubation for one hour in the dark. The oxidation was continued by adding 2% ascorbic acid, 1 M NaOH and alkaline phosphatase and incubation for 1 hour at 37°C in the dark. The sample was acidified by 2 M HCl and filtrated in a centrifugal filter device (Millipore) at 4'000 x g for 20 minutes. The concentrations of biopterin and neopterin were determined reversed-phase HPLC and fluorimetric detection.

Immunoquantification by Western blot

A small portion of protein lysate from PAH assay was immediately flash-frozen after protein extraction and stored at -80°C until immunoblotting. Between 5 and 50 µg of lysate from PAH activity assay were used for verifying PAH expression in the various cell lines by Western blotting, using commercially available anti-PAH antibody PH8 (sc-58398, 1:300 dilution, Santa Cruz Biotechnology, USA or ab21170, 1:5000 dilution, Abcam, UK). In order to detect FLAG- or Myc-tagged PAH proteins, monoclonal anti-FLAG M2 antibody (F1804, 1:1000, Sigma) and c-Myc antibody (sc-40, Santa Cruz Biotechnology) were used. Secondary antibodies used were ECL mouse and rabbit IgG, HRP-linked (1:10000 dilution, NA931V, NA934V, GE Healthcare, CT, USA). The antibody used for β-actin detection (AC-74, Sigma) was applied in a 1:10000 dilution.

Antibody binding was detected by enhanced chemiluminescence (Amersham ECL™, GE Healthcare) as described by the manufacturer's instructions.

Stability of PAH proteins after cell lysis

The stability of PAH proteins after cell lysis was assessed by immunoblotting. A small portion of total protein lysate from PAH activity assay was frozen in liquid N_2 immediately

after cell lysis and protein extraction. Subsequently, another portion of protein was frozen 1, 2, and 3 hours after cell lysis and storage on ice. These frozen protein lysates were stored at -80°C until immunoblotting.

Results and discussion

Selection of the transfection method and cell system

Hepatic and renal cell lines manifest a close approximation to the human *in vivo* environment of PAH expression, for which highest PAH activity is found in the liver and in the kidneys. Three human liver cell lines (HepG2, Hep3B and HuH-7) and one kidney cell line (COS-1, monkey origin) were tested and optimized for high transfection efficiency and PAH expression. This was a requirement on the cell system for specific measurements of PAH activity in each mutant and distinguishing between the mutants. However, the cell lines should not exhibit high endogenous PAH activity in order not to be confound with expressed PAH form the pCMV-FLAG-PAH vector. HepG2 cells were difficult to transfect due to clustering cell growth behavior. Before reaching confluency on the cell culture dish, cells rather grow in multilayers, less accessible for uptake of exogenous DNA. For this reason, the HepG2 cell line was excluded from the study. HuH-7 cells are fast growing, monolayer cells. The comparison of GFP-transfected HuH-7 cells with Jetpei, Pei200 and Fugene 6 reagents showed higher amounts of green-fluorescing cells with Pei200 up to 10 µg plasmid DNA compared to Jetpei reagent. However, cytotoxicity was high after 48 hours even though Pei200/DNA complexes were removed after 3 hours. Cytotoxicity was markedly reduced upon transfection of 10 µg DNA with Fugene 6, although with reduced efficiency compared to Pei200 reagent (Figure 5.3 Panel A).

Hep3B cells have been included in this study as they were already used in PAH expression studies by Aguado et al. (20) with the same PAH expression vector and Jetpei transfection reagent. They showed lower transfection efficiencies than found in HuH-7 cells upon GFP expression and slower cell growth. These results lead to the conclusion that HuH-7 is a more favorable cell system than Hep3B cells for the study of co-expression of PAH mutations.

In parallel to the optimization of the transfection protocol, PAH activities were measured in the various cell lines transfected with Jetpei, Pei200 and Fugene 6. The transfection protocols were therefore adapted from 6-well plates to 10-cm sized culture plates for higher protein expression. Figure 5.2 shows the comparison between PAH activities measured in Hep3B, HuH-7 and COS-1 cells. PAH activities detected in the hepatic cell lines were less than 0.1 mU/mg protein. As COS-1 cells have also been used in various PAH expression studies (3, 21), we decided to include this cell line in our study. DEAE/Dextran and Lipofectamine transfection protocols were already established and successfully used in the laboratory and were therefore tested with the COS-1 cell line. DEAE transfection

experiments showed almost no GFP expression. However, Lipofectamine transfections showed high GFP expression and higher PAH activity than found in HuH-7 cells (Figure 5.2, Figure 5.3).

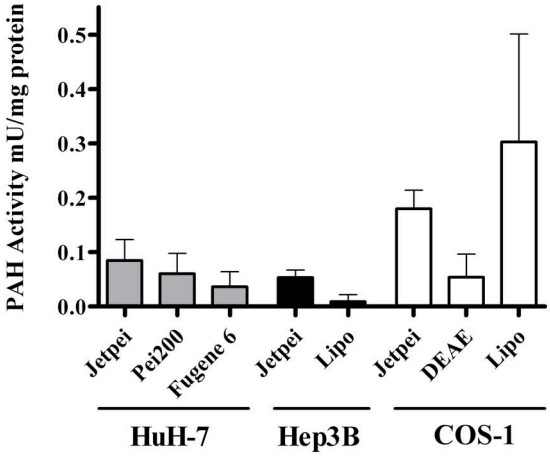


Figure 5.2: Comparison of PAH activities measured in three transfected mammalian cell lines and using different transfection methods. Lipo = Lipofectamine

PAH protein expression was also compared by immunoblotting and similar results were found than in PAH activity assays. Transfecting a higher DNA amount (20 µg) with Jetpei reagent led to slightly higher PAH protein expression. However, in this comparative analysis, transfecting 10 µg of plasmid vector with Pei200 led to highest PAH expression.

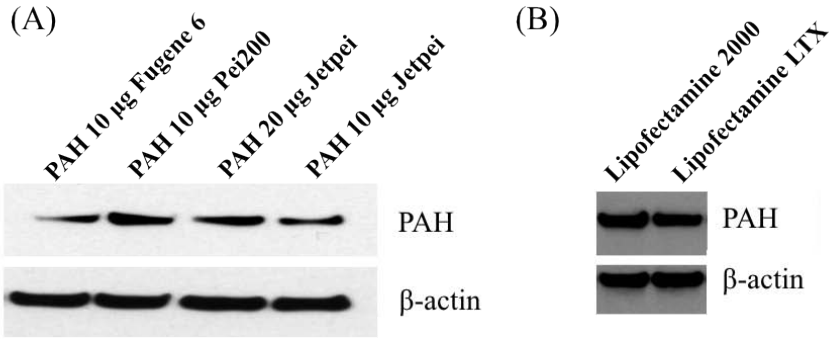


Figure 5.3: Comparison of PAH expression using anti-PAH PH8 antibody. (A) HuH-7 cells transfected with 10 or 20 µg pCMV-FLAG-PAH with Fugene 6, Pei200 or Jetpei transfection reagent. (B) COS-1 cells transfected with 12 µg pCMV-FLAG-PAH with Lipofectamine 2000 and LTX reagent. No PAH expression was detected in non-transfected HuH-7 or COS-1 cells.

High protein expression was found in transfected COS-1 cells with Lipofectamine 2000 and Lipofectamine LTX reagents. In addition, PAH wild type expression was found much higher in COS-1 cells than in liver cell lines. Figure 3.5 panel B in Chapter 3 of this thesis shows a protein expression comparison of wild type PAH transfected with the same protocol using Fugene 6 reagent in COS-1, Hep3B and HuH-7 cells. Even though less protein lysate from COS-1 cells was analyzed by immunoblotting, transfected PAH expression was visibly

higher in COS-1 cells than in the two hepatic cell lines. Fugene 6 transfection was found more efficient with serum-containing medium than with Opti-MEM reduced serum medium, and no medium change was necessary for 48 hours.

Fugene 6 is a transfection reagent with straightforward transfection protocol, leading to high PAH expression and little cell toxicity. In addition, a high measurable range of PAH activities is required for detection of significant differences between the various PAH mutants to be tested. COS-1 cells were concluded to be a suitable cell system for culturing and transfection with Fugene 6 reagent and fulfilled the requirements set for this co-expression study.

Stability of PAH protein lysates

We assessed the stability of PAH protein lysates extracted from transfected COS-1 cells upon storage on ice until the completion of PAH activity assay. As can be seen from Figure 5.4, PAH protein amounts remained comparable over a time course of at least 3 hours. This time is sufficient to complete a batch of PAH activity measurements. However, during the immunoblotting procedure, the transfer of the proteins to the membrane was sub-optimal, resulting in disturbed β-actin signals. Nonetheless, the signals do not show noticeable differences in protein amounts loaded.

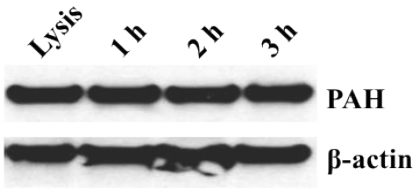


Figure 5.4: PAH wild type protein turnover transiently expressed in COS-1 cells after cell lysis for PAH activity assay. Anti-PAH PH 8 (Santa Cruz) antibody was used.

Optimization of PAH assay protocol

The expression plasmid containing CMV promoter in this study is used for constitutive high levels of gene expression. The cell line and transfection method were optimized with the same purpose of obtaining high PAH expression. However, the PAH activity range detected was still rather small and amino acids quantified were in the low micromolar range and signals of the amino acid analyzer were rather unspecific. This made the efficient separation of various PAH mutant activities cumbersome and unspecific. The PAH assay protocol might not be optimal and sensitive enough for PAH expression in mammalian cells, which contain lower protein amounts compared to mouse liver samples previously measured with this assay.

Tandem mass spectrometry displays lower limits of detection than the amino acid analyzer and is more specific for quantification of amino acids because of the use of internal isotope standards. The quantification of PAH activity was therefore transferred to a liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MSMS) machine. The PAH activity assay protocol was simultaneously adapted to the assay procedure described by Pey at al. (34). The successful optimization of the PAH assay protocol was described in detail in Chapter 3 of this thesis. All further PAH transfection experiments were performed in COS-1 cells using Fugene 6 transfection reagent with the optimized PAH assay protocol and resulted in 100 times higher PAH wild type activities (Chapter 3). This robust, highly specific and accurate method for the determination of PAH activities was used in all following experiments.

The LC-ESI-MSMS method was setup using only FLAG-tagged PAH proteins. Before co-expression of two variants in cells, PAH activity of identical mutations with either FLAG- or Myc-tagged proteins were compared. No significant differences in activity due to a different epitope tag could be detected.

Co-expression of PAH p.R261Q with p.R158Q

The single expression of several common PAH mutations has been performed for the validation of the LC-MSMS method to compare activity assay results with other methods for validation. With single expression results known, the co-expression of two distinct PAH alleles in COS-1 cells was initiated in a 1:1 ratio of identical amounts of each DNA vector. No differences in PAH activity were found between FLAG-p.R261Q and Myc-p.R261Q, as well as between FLAG-p.R158Q and Myc-p.R158Q in single expression. The co-transfection of p.R158Q with PAH wild type showed no reduction of activity (Figure 5.5).

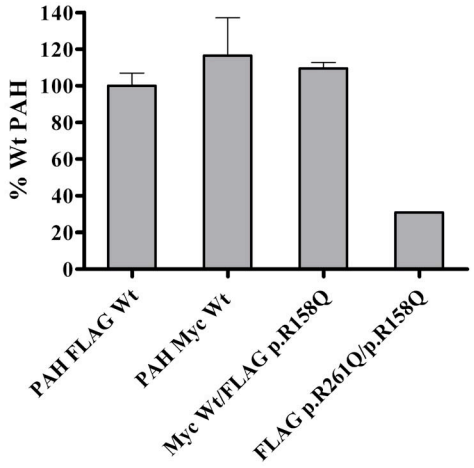


Figure 5.5: Residual activity expressed in percent (%) PAH wild type (Wt) of p.R158Q with PAH wild type and with p.R261Q. Residual activities were normalized for transfection efficiency.

The p.R158Q mutation is a severe mutation with low residual PAH activity (5% of the wild type activity), but high amounts of mutant protein were detected (see Figure 3.3 panel C). The co-expression of p.R158Q with PAH wild type did not result in a residual activity of 54%, as calculated by averaging the activity of wild type and p.R158Q. The measured residual activity does not seem markedly influenced by the severe p.R158Q mutation. However, the co-expression of p.R261Q/p.R158Q showed 30% of residual PAH activity and was slightly higher than predicted 24% from averaging single expressed PAH activity for each mutant. This genotype was reported in mild and classic PKU patients and responsive to supplementation of BH_4 (9, 35). The response to BH_4 in cell culture remains to be tested. However, these results showed that it is possible to assess residual PAH activity of two distinct, co-transfected PAH mutants.

Treatment of transfected COS-1 cells with BH_4

BH_4 supplementation to transfected cells was used to study BH_4 responsiveness, resulting in increased residual PAH activity for some of the mutants investigated. Figure 5.6 shows the results of PAH activity and pterin measurements in COS-1 cells transfected with PAH p.R261Q. The p.R261Q mutation is frequently reported with inconsistent BH_4 responsiveness. The effect of BH_4, ascorbic acid (Vitamin C) or the combination of BH_4/Vitamin C on residual PAH activity was assessed. Panel A depicts PAH activities normalized to PAH wild type using β-gal assay results for transfection efficiencies. Vitamin C is a stabilizing agent of BH_4 and should not have an effect on PAH activity. As can be seen in panel A, PAH p.R261Q displays the same activity (40% PAH wild type) with and without Vitamin C treatment. However, PAH activity after treatment of the cells with BH_4 or BH_4/Vitamin C is markedly reduced. In panel B, the absolute PAH activities, which are not normalized for transfection efficiency, are displayed for the various treatment conditions.

It was noted, that absorbance reads from β-gal assay in BH_4-treated lysates were only 10% of non-treated cells or cells treated with Vitamin C only and were below the linear range. The treatment by BH_4 apparently delays β-gal assay reaction as absorbance reads only reach comparable values to non-treated cells after longer incubation times. The results displayed in panel A were therefore correlated with time of incubation in the β-gal assay at 37°C. However, they are not conclusive for the effect of BH_4 on β-gal assay and therefore we compared normalized PAH activities with absolute activities in panel B. The absolute activities are similar in all samples whether treated with BH_4, Vitamin C or a combination of both. Under assumption that transfection efficiencies are similar in all cell dishes, BH_4 and

Vitamin C treatment does not result in increased PAH activities under conditions used, but shows rather lower PAH p.R261Q activity.

After 24 hours of treatment with BH_4 a small sample of cell culture medium was removed for measurement of pterin concentrations to analyze whether Vitamin C stabilizes BH_4 in cell culture and whether BH_4 is consumed rapidly. Fresh culture medium samples supplemented with the initial concentration of 75 µM BH_4 was included for comparison. In medium without Vitamin C and BH_4, no pterin was detected and little amounts of biopterin (6.2 nmol/L). In all other samples, biopterin concentrations were below 600 nmol/L but high pterin concentrations were detected (24'000 - 29'000 nmol/L). However, these samples were not processed by differential oxidation, and it cannot be concluded on total BH_4 amounts.

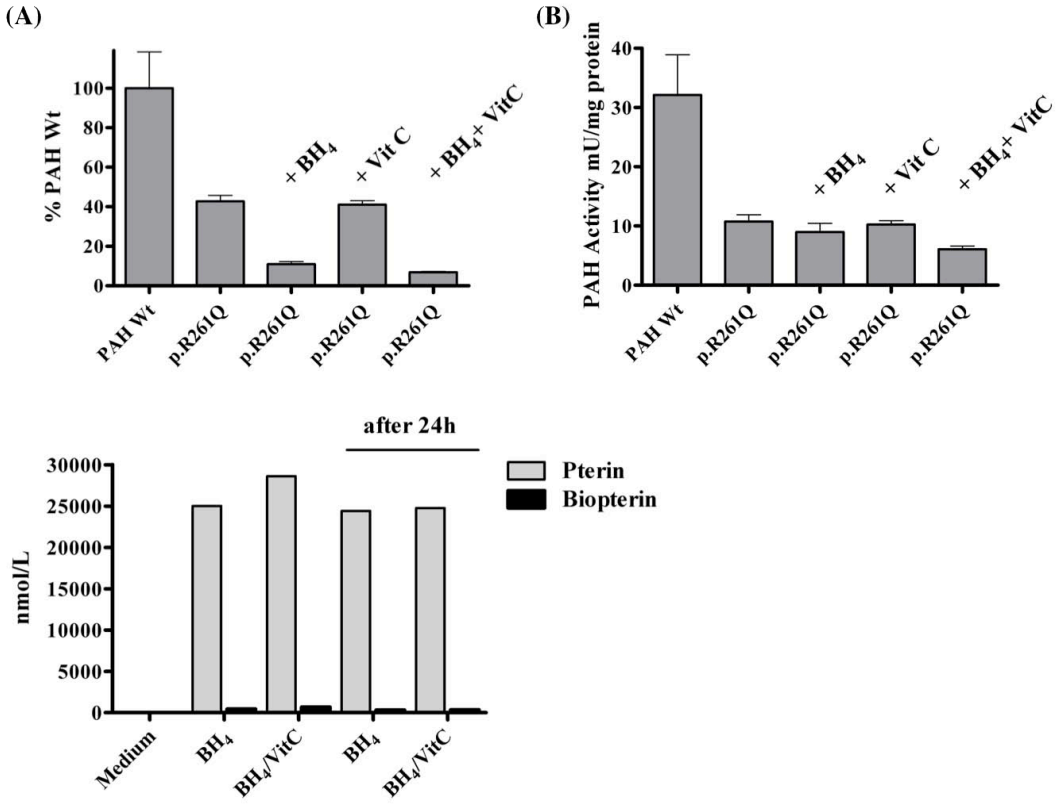


Figure 5.6: Treatment of p.R261Q transfected COS-1 cells with BH_4 and ascorbic acid and analysis of the stability of BH_4 in cell culture. (A) Normalized PAH activities (B) Non-normalized absolute PAH activities (C) Comparison of BH_4 in fresh cell culture medium or after 24 hours incubated on cells at 37°C, with or without stabilization with ascorbic acid (Vitamin C, VitC). These are results from a single experiment and measurements in duplicate. Medium: DMEM without supplementation of BH_4

BH_4 is stable in acidic solution, but at basic pH, BH_4 is readily oxidized to pterin (2-amino-4-hydroxy-pteridine, removal of BH_4 side chain) (31). The pH of DMEM cell culture medium is neutral to slightly basic. It could be speculated that the basic pH of medium and culturing at 37°C are sufficient to degrade BH_4 to pterin, but a conclusion on the effect of Vitamin C on BH_4 cannot be drawn from these results.

Treatment of responsive PAH p.E390G with BH_4

The PAH p.E390G mutation has been reported as BH_4-responsive, independent of the second allele (36). In the following experiment the activity of p.E390G with FLAG-tag and with Myc-tag was compared. All the FLAG-tagged cell lysates were stopped at the same time point during the β-gal assay to avoid incoherence with β-gal assay results. Both Myc-tagged samples were gathered at an earlier time point because of increased β-gal activity in the samples. Figure 5.7 shows the effect of BH_4 on PAH p.E390G activity with an increase in activity of 30%.

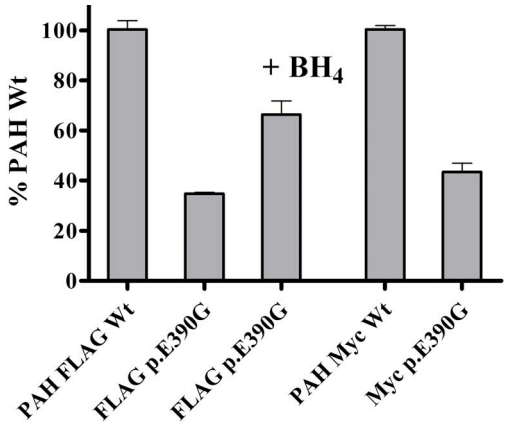


Figure 5.7: Effect of treatment of FLAG-PAH p.E390G with BH_4 on residual PAH activity. The FLAG-tagged constructs were normalized to FLAG-wild type (Wt) for transfection efficiency and Myc-tagged p.E390G construct to Myc-Wt. Results are from a single experiment with duplicates measurement.

However, PAH wild type transfected cells were not treated with BH_4 and the effect of BH_4 and p.E390G cannot be readily compared.

Comparison of the effect of BH_4 and sepiapterin treatment on PAH p.R261Q

Sepiapterin has been used to assess BH_4 responsiveness in cell culture (20). After entering the cell, sepiapterin is reduced to 7,8-dihydropterin (BH_2) via the salvage pathway by SR. BH_2 is a substrate for DHFR, which catalyzes the subsequent reduction to BH_4. Figure 5.8 panel A shows a 50% increase in PAH p.R261Q activity with BH_4, as well as with BH_4 stabilized by Vitamin C and by sepiapterin supplementation. The cells transfected with wild type plasmid were not treated with BH_4 or sepiapterin. A 70% increase in PAH activity upon sepiapterin treatment of non-transfected hepatoma cells has been reported by Aguado et al. (20), as well as 10% increase in PAH protein expression in transfected hepatoma cells.

The effect of BH_4, Vitamin C and sepiapterin on wild type PAH activity was not assessed in this experiment. Under the assumption that wild type PAH activity increases upon BH_4 treatment in transfected COS-1 cells, as described in (20) for hepatoma cells, the activity of

PAH p.R261Q in response to BH_4 or sepiapterin would not exceed wild type PAH activity. The β-gal assay results were lower in sepiapterin-treated cells compared to non-treated cells, although the decrease was not as dramatic as with BH_4 treatment. Therefore β-gal assay samples could still be harvested at the same time point without being out of the linear range of β-gal activity.

However, the absolute PAH activity results in panel B of Figure 5.8 confirm the data from Figure 5.6, showing that BH_4/Vitamin C treatment does not lead to increased residual activity.

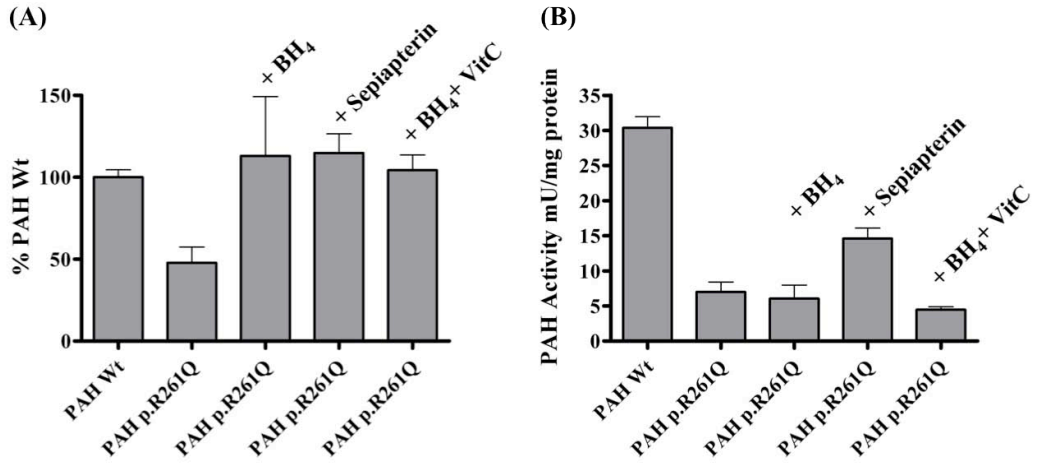


Figure 5.8: Comparison of PAH p.R261Q response to treatment with BH_4 and with sepiapterin. (A) Mutant activities were normalized for transfection efficiency and expressed as % wild type. (B) Absolute PAH activities are depicted, not normalized for transfection efficiency. All PAH p.R261Q constructs were gathered at the same time point in β-gal assay and the time factor was included for normalization to PAH wild type (Wt). Results are from a single experiment with duplicates measurement.

However, the absolute PAH activity results in panel B of Figure 5.8 confirm the data from Figure 5.6, showing that BH_4/Vitamin C treatment does not lead to increased residual activity. In order to verify whether BH_4 and sepiapterin are influencing the *in vitro* β-gal assay leading to lower absorbances and not influencing transfection efficiency, we transfected COS-1 cells with only pSV-βgal plasmid and treated one cell plate each with BH_4, sepiapterin, and BH_4/Vitamin C. After 48 hours, cells were stained using X-gal solution from in-situ staining kit. No differences in transfection efficiencies were observed from the stained COS-1 cells. BH_4 and sepiapterin are oxidizing agents and may indeed influence β-galactosidase enzyme or assay components.

Stability of sepiapterin in cell culture

Sepiapterin is less sensitive to oxygen than BH_4, but it is more sensitive to light. It is known that exogenously administered sepiapterin is efficiently incorporated into cells where

it is reduced to BH_4 via the salvage pathway (37, 38). From a previous work (39), we know that sepiapterin solution is stable at 37°C in the dark, which is comparable to the condition in a cell culture CO_2 incubator (Figure 5.9).

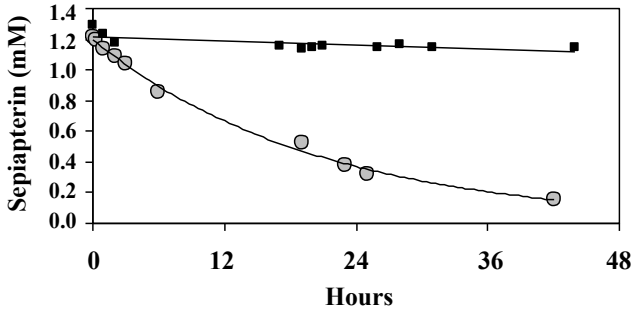


Figure 5.9: Decomposition of 1.25 mM sepiapterin stock solution when left at daylight and room temperature: ■ sepiapterin solution stored in the dark at 37°C; ○ sepiapterin solution kept at daylight and room temperature. [SP] measured in 1:20 dilution at 420 nm, ε_λ = 10400 M^{-1} cm^{-1}. (Figure from (39)).

To investigate the intracellular conversion of sepiapterin to BH_4, we have measured pterin concentrations in COS-1 cell lysates that were transfected or not transfected with PAH wild type or p.E390G (Figure 5.10). Biopterin concentrations were compared between non-treated and sepiapterin-treated cells. The cell lysates used for PAH activity assay were oxidized by iodine under acidic and alkaline conditions to differentiate between BH_4 and biopterin and measured by HPLC. No biopterin could be detected in non-treated cells. Biopterin concentration was highest in the p.E390G transfected cells, a known BH_4-responsive mutation. Pterin concentrations were only slightly increased after sepiapterin treatment.

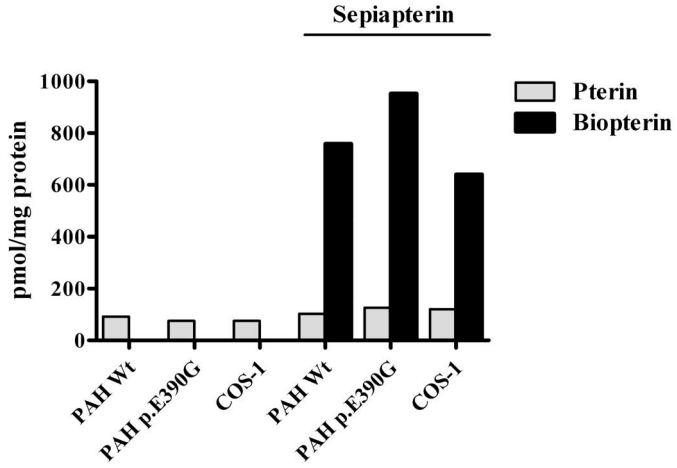


Figure 5.10: Pterin and biopterin concentrations in cell lysates before and after treatment with sepiapterin, processed by differential oxidation.

The biopterin concentration determined in this experiment is the total concentration after acidic and alkaline oxidation of the cell lysates. BH_4, qBH_2, and BH_2 are oxidized to biopterin in the first step of the differential oxidation process under acidic pH, which

suggests that the cells take up sepiapterin and metabolize it. Pterin results from BH_4 oxidation after alkaline oxidation. As the increase in pterin after treatment is only minor, it can be concluded that only a small amount of BH_4 was present in these cell lysates. However, no conclusions can be drawn from this experiment regarding the amount of sepiapterin used by PAH wild type and p.E390G and whether one of the constructs would use more BH_4 than the other constructs.

Further measurements in an identical experiment would be necessary to determine the amount of sepiapterin in cell medium at the moment of addition and after 43 hours of cell culture at 37°C. The stability of sepiapterin should also be tested at lower concentration than shown in Figure 5.9. These results would have to be deducted from the sepiapterin concentration in cell medium.

Effect of sepiapterin on PAH wild type

In the report by Aguado et al. (20) a 10% increase in PAH wild type protein was demonstrated after treatment of transfected Hep3B cells with 100 µM sepiapterin. The PAH activity after the treatment was not determined, which is difficult to distinguish from endogenous PAH activity. However, they reported no increase in mRNA expression levels in the hepatoma cells, and a 70% higher PAH activity in the non-transfected cells. The amount of PAH protein increased by 270% after treatment with increased half-life and increased biopterin levels were found.

We measured the PAH wild type activity expressed in COS-1 cells and supplemented with 75 µM sepiapterin for 43 hours. PAH activity was increased by 80% after normalization to β-gal activity, as shown in Figure 5.11.

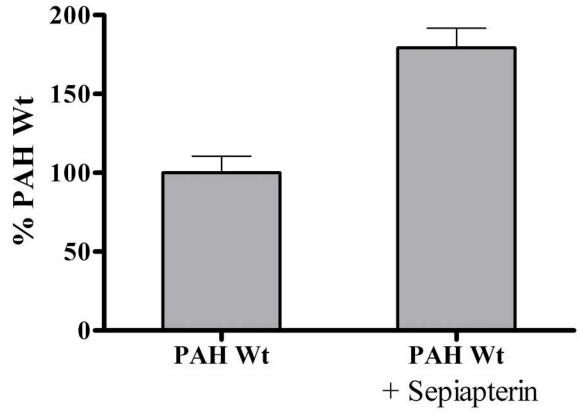


Figure 5.11: Effect of sepiapterin treatment on transfected COS-1 cells with PAH wild type (Wt). Results are from a single experiment with duplicates.

Effect of sepiapterin and phenylalanine on PAH p.R261Q

The phenotype associated with p.R261Q/p.R261Q is mostly a mild PKU phenotype. However, also some classic PKU patients were reported with this genotype (16, 40). As PAH is tightly regulated by BH_4 and Phe, the effect of both compounds on the PAH p.R261Q residual activity was assessed. The cells were treated with 75 µM sepiapterin or 1200 µM Phe, mimicking a classic PKU environment.

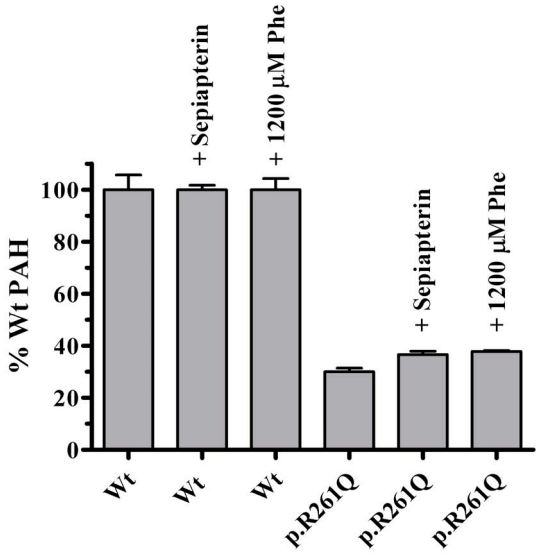


Figure 5.12: Comparison of PAH p.R261Q treated with 75 µM sepiapterin or 1200 µM Phe. The residual activity has been normalized to the PAH wild type (Wt) treated identically. Results are from a single experiment with duplicates measurement.

Sepiapterin and Phe treatment of p.R261Q resulted in slight PAH activity increases. β-gal activity results were found again decreased upon sepiapterin treatment for both constructs. It is therefore recommended to normalize PAH mutant proteins only to identically-treated PAH wild type.

Co-expression of p.R261Q/p.R408Q and effect of BH_4 and phenylalanine treatment

Initially, the PAH activities of p.R261Q and p.R408Q were compared after single and co-expression in COS-1 cells. p.R408Q has been reported as mild mutation with high residual activity (55%), but found in a mild or classical PKU phenotype, which was explained by an influence on splicing regulation on mRNA level of this mutation (35, 41, 42). We found a residual activity of 22% for p.R408Q, which may vary depending on the cell type used for *in vitro* expression and amount of wild type transcript expressed.

The co-expression of p.R261Q/p.R408Q showed residual activity of 28% (predicted residual activity from the single expression: 31%). The treatment with sepiapterin showed an increase of 55% activity for this genotype.

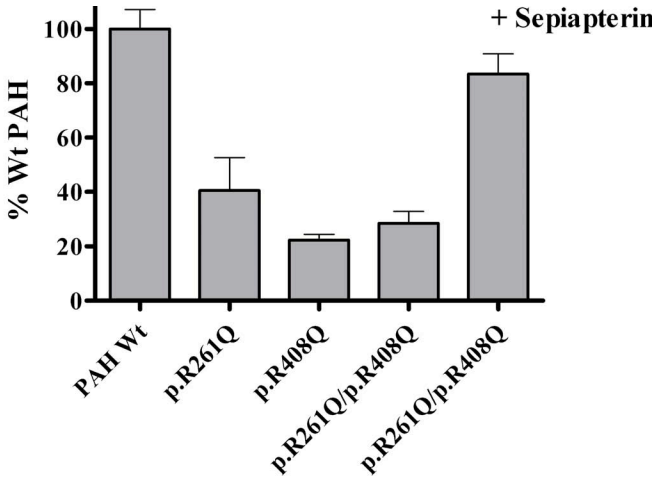


Figure 5.13: Comparison of single and co-expression of PAH p.R261Q and p.R408Q in COS-1 cells and treatment with sepiapterin. Results are from a single experiment with duplicates measurement.

PAH wild type has not been treated with sepiapterin in this experiment. If we speculate that wild type activity always increases by 70% after treatment and re-normalize the results, the increase in activity for p.R261Q/p.R408Q would only be 5%. In addition, the increase of PAH protein amount after treatment should be considered for a final conclusion on BH_4 responsiveness with this genotype. Previously, high p.R408Q protein expression was reported (21, 42). Therefore, sepiapterin treatment may favor the stabilization of p.R408Q full mRNA transcript and therefore lead to an increase in protein expression and activity.

The treatment of the transfected cells with Phe led to a 15% increase of residual p.R261Q/p.R408Q activity and the combined sepiapterin/Phe treatment to an increase of 13%. The results were normalized to the non-treated PAH wild type. PAH expression analysis by Western blot (still pending) will show whether an increase in protein expression led to the increase in residual activity. However, sepiapterin treatment does not seem to be effective at high Phe levels, which corresponds to the classic phenotype. Further experiments are needed to conclude on BH_4-sensitivity of this genotype.

In the literature, the p.R408Q mutant was reported with higher affinity for the substrate than wild type PAH (4). In addition, p.R408Q expression was found increased in a co-overexpression with chaperonin GroESL, pointing to a susceptibility for response to BH_4. Only one patient has been documented in the BIOPKU database carrying this genotype corresponding to a mild phenotype and non-responsive to BH_4.

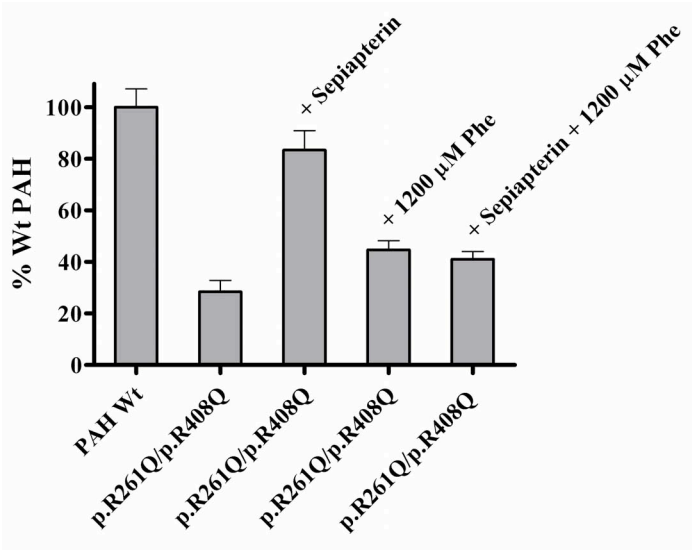


Figure 5.14: Comparison of co-expression of PAH p.R261Q and p.R408Q upon supplementation with sepiapterin or Phe or a combination of both reagents. Results are from a single experiment with duplicates measurement.

Co-expression of p.R261Q/p.I65T and effect of sepiapterin supplementation

The co-expression of p.R261Q and p.I65T is an example of negative interaction of two distinct PAH subunits. The predicted residual activity for this genotype combination is 38% (measurements with this system). The co-expression in COS-1 cells revealed 19% PAH activity, similar to the results reported by Leandro et al. (15) in a bacterial co-expression system of recombinant hPAH subunits. The supplementation of sepiapterin led to a 20% increase in residual activity.

Three patients with this genotype have been reported in the BIOPKU database with Phe-levels at diagnosis above 850 µM. Only one responder was found, who had normal Phe-levels at the time of BH_4-loading test. The other two patients responded slowly or were non-responder in a combined Phe/BH_4-loading test.

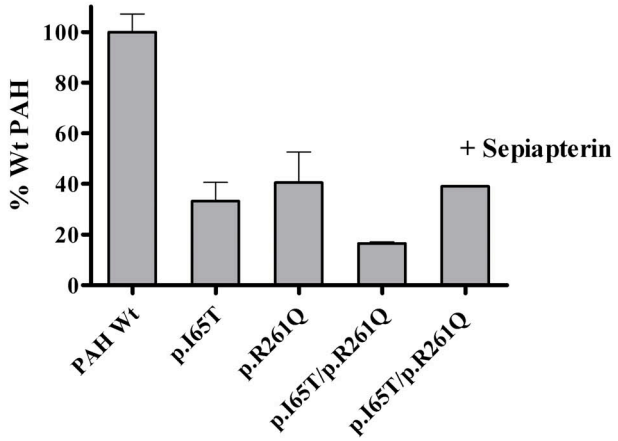


Figure 5.15: Co-expression of PAH p.I65T and p.R261Q and effect of sepiapterin treatment in COS-1 cells. Results are from a single experiment with duplicates measurement.

Both mutations lie at the dimer interface. The p.I65T mutation is predicted to result in a significant structural perturbation of the regulatory core domain (43). *In vitro* residual activity was found increased in a Phe-activated assay setup for PAH compared to non-activated PAH, and with reduced affinity for the cofactor (2). p.R261Q lies in a cofactor binding region and increased concentrations of cofactor restore PAH activity. Therefore, we predicted that in a phenotype with high Phe levels, the combination of conformational correction by the substrate and p.R261Q response to the cofactor results in higher PAH activity. However, the interplay of high Phe and BH_4 treatment remains to be tested in this mammalian co-expression cell system.

Conclusions

The frequent inconsistencies in genotype-phenotype correlations and inconsistent reports of response to BH_4 for several mutations constitute disadvantages in the fast and efficient genotype-based diagnosis and adjusting of tailored treatment in PKU patients. Although the genotype is the main determinant of the phenotype and as is now generally accepted as a valuable determinant for BH_4 responsiveness, genotyping does not always provide conclusive prediction. In 1975, Kaufman reported on the phenomenon of negative interallelic complementation in PKU patients for illustrating protein-protein interactions between the subunits of multimeric human PAH enzymes (44). It was often assumed from patient investigations that mild alleles with high activity dominate over alleles with low activity (42, 45), but direct evidence was difficult to obtain. Two-hybrid systems for example demonstrated that two PAH subunits interact with each other and form heteromeric proteins. However, available *in vitro* expression systems generally did not allow the study of the impact of a particular mutation on PAH activity in the heteromeric enzyme and assessment of BH_4 responsiveness of the investigated genotype.

A number of PAH mutations listed in the BIOPKU database were associated with BH_4 responsiveness, but the influence of the other allele in the *in vivo* situation can be crucial. In the present chapter, various experiments were described aiming at establishing and validating a co-expression system of PAH mutants in mammalian cells. The evaluation of BH_4 sensitivity and effect of various Phe levels were analyzed by the co-expression of different PAH allele combinations. The combination of site-directed mutagenesis, co-expression in mammalian cells and relation to the influence of mutations on the three-dimensional structure represent a powerful tool for examining mutant protein residual activity. For time reasons, the variety of experiments was increased in order to evaluate the system but repetitions for strengthening conclusions on the obtained results and establishing statistical significances were postponed. The two expression vectors differ only in a few base pairs and are therefore expected to transfect and express PAH cDNA in similar efficiencies. However, protein quantification needs to be performed for every expression experiment and if divergent for both mutants, it should be correlated with residual activity. Especially if BH_4 supplementation to a particular mutant leads to increased protein expression, the analysis of protein expression hints to stabilization by BH_4.

The PAH proteins were fused to epitope tags in order to confirm and compare expression levels of each protein and determine a contribution of both mutants to residual PAH activity. In addition, the tags allow the study of protein interactions between the two mutants by co-

immunoprecipitation. The subunits of each dimer contact and interact with the adjacent dimer. It was planned to purify Myc-tagged PAH proteins using a commercially available kit and immunoblot with anti-FLAG antibody. Under the assumption that interactions are strong enough to resist precipitation, the blot would have shown interactions by the two co-expressed PAH mutants. In addition, the influence of the epitope tag on PAH activity remains to be evaluated, as well as the influence of the location of the tag. PAH mutant subunits should also be produced with C-terminally tagged proteins and co-expressed. The same range of activity for each mutant should result.

As PAH is tightly regulated by the concentrations of Phe and BH_4 (18, 46), both compounds need to be considered when assessing BH_4 responsiveness. The co-expression system allows the fast determination of residual PAH activity in combination with presence or absence of BH_4 and high or normal Phe levels. BH_4-loading test protocols are not harmonized throughout the world and variation of Phe intake during the test often leads to inconsistent results. BH_4-response may vary if a patient is on dietary restriction or not, as Phe levels interact with response and it is the ratio of BH_4 and Phe that determines activation and inhibition of PAH. Some PAH mutants even reach their peak activity at higher substrate concentrations (19). Increased Phe levels may lead to increased enzyme activity and also favor conformational rearrangements promoting access of the cofactor (increased affinity of the mutant for the cofactor). Eventually only then an effect of BH_4 in the more severe phenotypes is observed. Nevertheless, treatment by sapropterin facilitates diet compliance, increases Phe tolerance, and should be favored if possible. The previous evaluation in cell culture and comparison with a genotype database enables the development of a recommendation on BH_4 responsiveness and may even prevent some patients from performing the loading test.

The choice of BH_4 or sepiapterin formulation and concentration was made considering previous studies (19, 20). The biosynthesis of BH_4 was investigated in COS-1 cells (47). However, information on the presence of dihydrofolate reductase activity in this cell line, in order to synthesize BH_4 from sepiapterin, was missing. For this reason, both BH_4 and sepiapterin were tested in COS-1 cells. Hasegawa et al. (37) demonstrated that several cultured cell lines take up sepiapterin more effectively than BH_4 resulting in increased intracellular BH_4 concentrations. The intracellular BH_4 pool was found stable after sepiapterin supplementation. In addition and as already mentioned, supplemented BH_4 is readily oxidized to BH_2 in the extracellular fluid (37). These data, together with the stability of sepiapterin at 37°C and less influence on β-gal activity, led to the conclusion that

sepiapterin supplementation is favorable, resulting in stable intracellular BH_4 concentrations over the time of transfection. Moreover, in further experiments, Phe and BH_4 concentrations in cell culture could be varied to study the detailed concentration dependence of these reagents. The time of addition after transfection to the cell culture medium may influence residual PAH activity as well and should be further investigated.

Correlations of the genotype and prediction of BH_4 responsiveness remain complex as long as there is no gold standard test for assessing sensitivity to BH_4 and inconsistent results persist. However, the co-expression system described herein can be used for the analysis of many genotype combinations under various culturing conditions. The investigation of many different allele combinations will allow the establishment of dominance effects for each mutation and algorithms for the prediction of BH_4 responsiveness.

Supplementary Table

Primer sequences, used for site-directed mutagenesis generating PAH mutants (Table 5.2) for *in vitro* expression:

Primer name	Sequence 5' → 3'
PAH p.R261Q fwd	ggcctggccttcc**a**agtcttccactgc
rev	gcagtggaagact**t**ggaaggccaggcc
PAH p.R408W fwd	ggaactttgctgccacaatacct**t**ggcccttctcag
rev	ctgagaagggcc**a**aggtattgtggcagcaaagttcc
PAH p.E390G fwd	ccctctattacgtggcag**g**gagttttaatgatgccaa
rev	ttggcatcattaaaactc**c**ctgccacgtaatagaggg
PAH p.R158Q fwd	gtgtaccgtgcaagac**a**gaagcagtttgctgac
rev	gtcagcaaactgcttc**t**gtcttgcacggtacac
PAH p.I65T fwd	atgatgtaaacctgacccaca**c**tgaatctagaccttctc
rev	gagaaggtctagattca**g**tgtgggtcaggtttacatcat
PAH p.R408Q fwd	ctgccacaatacctc**a**gcccttctcagttcg
rev	cgaactgagaagggc**t**gaggtattgtggcag
PAH p.L48S fwd	cactcaaagaagaagttggtgcat**c**ggccaaagtattg
rev	caatactttggcc**g**atgcaccaacttcttctttgagtg
PAH p.E280K fwd	cccatgtatacccccc**a**aacctgacatctgcc
rev	ggcagatgtcaggtt**t**gggggtatacatggg
PAH p.Y414C fwd	ggcccttctcagttcgct**g**cgacccatac
rev	gtatgggtcg**c**agcgaactgagaagggcc

Corresponding nucleotide change is marked in **bold**.

References

[1] Bernegger, C. and Blau, N. (2002). "High frequency of tetrahydrobiopterin-responsiveness among hyperphenylalaninemias: a study of 1,919 patients observed from 1988 to 2002." Molecular genetics and metabolism 77(4): 304-313.

[2] Erlandsen, H., Pey, A. L., et al. (2004). "Correction of kinetic and stability defects by tetrahydrobiopterin in phenylketonuria patients with certain phenylalanine hydroxylase mutations." Proc Natl Acad Sci U S A 101(48): 16903-16908.

[3] Pey, A. L., Perez, B., et al. (2004). "Mechanisms underlying responsiveness to tetrahydrobiopterin in mild phenylketonuria mutations." Hum Mutat 24(5): 388-399.

[4] Perez, B., Desviat, L. R., et al. (2005). "Kinetic and stability analysis of PKU mutations identified in BH4-responsive patients." Molecular genetics and metabolism 86 Suppl 1: S11-16.

[5] Blau, N. and Erlandsen, H. (2004). "The metabolic and molecular bases of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency." Molecular Genetics and Metabolism 82(2): 101-111.

[6] Zurfluh, M. R., Zschocke, J., et al. (2008). "Molecular genetics of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency." Human mutation 29(1): 167-175.

[7] Blau, N., Hennermann, J. B., et al. (2011). "Diagnosis, classification, and genetics of phenylketonuria and tetrahydrobiopterin (BH4) deficiencies." Molecular genetics and metabolism 104 Suppl: S2-9.

[8] Trefz, F. K., Scheible, D., et al. (2009). "Significance of genotype in tetrahydrobiopterin-responsive phenylketonuria." Journal of inherited metabolic disease 32(1): 22-26.

[9] Leuzzi, V., Carducci, C., et al. (2006). "The spectrum of phenylalanine variations under tetrahydrobiopterin load in subjects affected by phenylalanine hydroxylase deficiency." Journal of inherited metabolic disease 29(1): 38-46.

[10] Kaufman, S. (1976). Phenylketonuria: Biochemical Mechanisms. Advances in Neurochemistry. B. W. Agranoff and M. H. Aprison. New York, Plenum Press**:** 1-132.

[11] Crick, F. H. and Orgel, L. E. (1964). "The Theory of Inter-Allelic Complementation." Journal of molecular biology 8: 161-165.

[12] Leandro, J., Leandro, P., et al. (2011). "Heterotetrameric forms of human phenylalanine hydroxylase: co-expression of wild-type and mutant forms in a bicistronic system." Biochimica et biophysica acta 1812(5): 602-612.

[13] Gamez, A., Perez, B., et al. (2000). "Expression analysis of phenylketonuria mutations. Effect on folding and stability of the phenylalanine hydroxylase protein." J Biol Chem 275(38): 29737-29742.

[14] Waters, P. J., Scriver, C. R., et al. (2001). "Homomeric and heteromeric interactions between wild-type and mutant phenylalanine hydroxylase subunits: evaluation of two-hybrid approaches for functional analysis of mutations causing hyperphenylalaninemia." Molecular genetics and metabolism 73(3): 230-238.

[15] Leandro, J., Nascimento, C., et al. (2006). "Co-expression of different subunits of human phenylalanine hydroxylase: evidence of negative interallelic complementation." Biochimica et biophysica acta 1762(5): 544-550.

[16] Blau, N. BIOPKU: International Database of Patients and Mutations Causing Tetrahydrobiopterin-responsive HPA/PKU, http://www.biopku.org

[17] Thony, B., Auerbach, G., et al. (2000). "Tetrahydrobiopterin biosynthesis, regeneration and functions." The Biochemical journal 347 Pt 1: 1-16.

[18] Pey, A. L. and Martinez, A. (2005). "The activity of wild-type and mutant phenylalanine hydroxylase and its regulation by phenylalanine and tetrahydrobiopterin at physiological and pathological concentrations: an isothermal titration calorimetry study." Mol Genet Metab 86 Suppl 1: S43-53.

[19] Staudigl, M., Gersting, S. W., et al. (2011). "The interplay between genotype, metabolic state and cofactor treatment governs phenylalanine hydroxylase function and drug response." Human molecular genetics 20(13): 2628-2641.

[20] Aguado, C., Perez, B., et al. (2006). "Analysis of the effect of tetrahydrobiopterin on PAH gene expression in hepatoma cells." FEBS Lett 580(7): 1697-1701.

[21] Pey, A. L., Desviat, L. R., et al. (2003). "Phenylketonuria: genotype-phenotype correlations based on expression analysis of structural and functional mutations in PAH." Hum Mutat 21(4): 370-378.

[22] Scriver, C. R., Hurtubise, M., et al. (2003). "PAHdb 2003: what a locus-specific knowledgebase can do." Hum Mutat 21(4): 333-344.

[23] Thomas, M. and Klibanov, A. M. (2003). "Non-viral gene therapy: polycation-mediated DNA delivery." Applied microbiology and biotechnology 62(1): 27-34.

[24] Thomas, M., Lu, J. J., et al. (2005). "Full deacylation of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to mouse lung." Proceedings of the National Academy of Sciences of the United States of America 102(16): 5679-5684.

[25] Morimoto, K., Nishikawa, M., et al. (2003). "Molecular weight-dependent gene transfection activity of unmodified and galactosylated polyethyleneimine on hepatoma cells and mouse liver." Molecular therapy : the journal of the American Society of Gene Therapy 7(2): 254-261.

[26] Rebuffat, A. *Personal Communication.*

[27] Oppliger, T., Thony, B., et al. (1995). "Structural and functional consequences of mutations in 6-pyruvoyltetrahydropterin synthase causing hyperphenylalaninemia in humans. Phosphorylation is a requirement for in vivo activity." The Journal of biological chemistry 270(49): 29498-29506.

[28] Thony, B., Ding, Z., et al. (2004). "Tetrahydrobiopterin protects phenylalanine hydroxylase activity in vivo: implications for tetrahydrobiopterin-responsive hyperphenylalaninemia." FEBS letters 577(3): 507-511.

[29] Ledley, F. D., Grenett, H. E., et al. (1987). "Biochemical characterization of recombinant human phenylalanine hydroxylase produced in Escherichia coli." J Biol Chem 262(5): 2228-2233.

[30] Fiege, B., Ballhausen, D., et al. (2004). "Plasma tetrahydrobiopterin and its pharmacokinetic following oral administration." Molecular genetics and metabolism 81(1): 45-51.

[31] Fukushima, T. and Nixon, J. C. (1980). "Analysis of reduced forms of biopterin in biological tissues and fluids." Analytical biochemistry 102(1): 176-188.

[32] Blau, N., Thony, B., et al. (2001). Disorders of Tetrahydrobiopterin and Related Biogenic Amines. The Molecular and Metabolic Bases of Inherited Disease. S. C.R., D. Valle, A. L. Beaudetet al. New York, Mc Graw-Hill Professional.

[33] Elzaouk, L., Leimbacher, W., et al. (2003). "Dwarfism and low insulin-like growth factor-1 due to dopamine depletion in Pts-/- mice rescued by feeding neurotransmitter precursors and H4-biopterin." J Biol Chem 278(30): 28303-28311.

[34] Pey, A. L., Ying, M., et al. (2008). "Identification of pharmacological chaperones as potential therapeutic agents to treat phenylketonuria." J Clin Invest 118(8): 2858-2867.

[35] Spaapen, L. J. and Rubio-Gozalbo, M. E. (2003). "Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, state of the art." Molecular genetics and metabolism 78(2): 93-99.

[36] Karacic, I., Meili, D., et al. (2009). "Genotype-predicted tetrahydrobiopterin (BH4)-responsiveness and molecular genetics in Croatian patients with phenylalanine hydroxylase (PAH) deficiency." Mol Genet Metab 97(3): 165-171.

[37] Hasegawa, H., Sawabe, K., et al. (2005). "Delivery of exogenous tetrahydrobiopterin (BH4) to cells of target organs: role of salvage pathway and uptake of its precursor in effective elevation of tissue BH4." Molecular genetics and metabolism 86 Suppl 1: S2-10.

[38] Nichol, C. A., Lee, C. L., et al. (1983). "Biosynthesis of tetrahydrobiopterin by de novo and salvage pathways in adrenal medulla extracts, mammalian cell cultures, and rat brain in vivo." Proceedings of the National Academy of Sciences of the United States of America 80(6): 1546-1550.

[39] Zurfluh, M. R. (2008). Metabolism and Regulation of Tetrahydrobiopterin and its Implications for BH4-Responsive Hyperphenylalaninemia and BH4-Deficiencies. Zurich, University of Zurich: PhD Thesis.

[40] Dobrowolski, S. F., Heintz, C., et al. (2011). "Molecular genetics and impact of residual in vitro phenylalanine hydroxylase activity on tetrahydrobiopterin responsiveness in Turkish PKU population." Mol Genet Metab 102(2): 116-121.

[41] Ellingsen, S., Knappskog, P. M., et al. (1999). "Diverse PAH transcripts in lymphocytes of PKU patients with putative nonsense (G272X, Y356X) and missense (P281L, R408Q) mutations." FEBS Lett 457(3): 505-508.

[42] Svensson, E., Eisensmith, R. C., et al. (1992). "Two missense mutations causing mild hyperphenylalaninemia associated with DNA haplotype 12." Human mutation 1(2): 129-137.

[43] Erlandsen, H., Patch, M. G., et al. (2003). "Structural studies on phenylalanine hydroxylase and implications toward understanding and treating phenylketonuria." Pediatrics 112(6 Pt 2): 1557-1565.

[44] Kaufman, S., Max, E. E., et al. (1975). "Phenylalanine hydroxylase activity in liver biopsies from hyperphenylalaninemia heterozygotes: deviation from proportionality with gene dosage." Pediatric research 9(8): 632-634.

[45] Avigad, S., Kleiman, S., et al. (1991). "Compound heterozygosity in nonphenylketonuria hyperphenylalanemia: the contribution of mutations for classical phenylketonuria." American journal of human genetics 49(2): 393-399.

[46] Gersting, S. W., Staudigl, M., et al. (2010). "Activation of phenylalanine hydroxylase induces positive cooperativity toward the natural cofactor." J Biol Chem 285(40): 30686-30697.

[47] Elzaouk, L., Laufs, S., et al. (2004). "Nuclear localization of tetrahydrobiopterin biosynthetic enzymes." Biochimica et biophysica acta 1670(1): 56-68.

Concluding Remarks

Since the discovery of PKU as a treatable genetic disease and the prevention of the harmful effects by a low-Phe diet, it is often described as a paradigm, which caused a shift in medical thinking about inherited disorders (1). The initiation of newborn screening and its later extension to other disorders is a great benefit for patients. Moreover, PKU constitutes a paradigm for protein misfolding diseases with a loss-of-function pathogenic mechanism (2). Still, PKU is a heterogeneous disease and highly complex at its enzymatic, metabolic and cognitive levels.

The molecular bases of PKU and their implications at the metabolic level with focus on BH_4 responsiveness were addressed in the present thesis. Results from initial patient-based studies were investigated in the laboratory by *in vitro* analysis and characterization of PKU mutations, including the establishment of a novel method tandem mass spectrometry method for assessing *in vitro* PAH activity.

In the first two chapters of this thesis, a large-scale assessment of Turkish and Croatian PKU patients with critical examination of genotypes and response to BH_4 challenge is described. The Croatian patients were selected for BH_4-loading tests solely based on the type of mutation. However, the prevalence of BH_4-responders among the group was lower than predicted and led to the conclusion that the complete genotype needs to be considered for more consistent prediction of BH_4 responsiveness. The Turkish PKU patients' dataset was larger and included the full genotype with *in vitro* residual PAH activities of both alleles and data from the BH_4 challenge. A substantial *in vitro* residual activity was found to be required for BH_4-response. Also, a high rate of homozygous genotypes was detected and in this group, BH_4 responsiveness was also related to substantial residual PAH activity. The detection of 23 novel *PAH* mutations highlights the importance of genotyping for better characterization of the phenotypes and heterogeneity in PKU disease.

Moreover, the Turkish patient study revealed 19 patients with elevated Phe levels where no *PAH* mutations were detected. The DNA was further assessed for mutations in *QDPR*, *PTS,* and *PCD* genes. Four different *QDPR* mutations were identified, as well as three *PTS* and one *PCD* mutation. The details of these results will be described in a separate follow-up study. However, the Turkish study contributed to the correct diagnosis of a BH_4-deficiency in these patients, as they require a different therapy than the low-Phe diet.

In some cases only one mutant *PAH* allele was detected and some of these samples will be further assessed by MLPA analysis or next-generation sequencing. A second mutation leading to a PKU phenotype may be deeply buried in an intron, which is not assessed in conventional genotyping methods. Lymphoblast cell cultures were initiated from the patients'

blood. They will be analyzed for aberrant mRNA splicing as well as sequenced using 454 sequencing.

The crystal structure of PAH is a valuable frame of reference in the molecular characterization of the large number of PAH mutations. It is still only a model, but in combination with *in vitro* expression studies, both provide valuable information for characterization of the mutations, the relation to the phenotype and prediction of BH_4 responsiveness. Several different *in vitro* PAH assay protocols have been reported in the literature with divergent results for identical mutations. Most of the time PAH activities are reported as percentage of the wild type. Quantification and detection limits, as well as coefficients of variation are generally not reported. The measurement of PAH *in vitro* activity is technically relatively difficult as two of the assay components, BH_4 and oxygen, react with each other in the absence of enzyme.

Chapter 3 focuses on the successful application of a LC-ESI-MSMS method for the quantification of Phe and Tyr in cell lysates and mouse tissues. The PAH activity assay with amino acid analyzer, as described in Chapter 5 and previously applied in this laboratory, was not sensitive enough to quantify Phe and Tyr in cell lysates. Therefore, a LC-ESI-MSMS method for the quantification and subsequent calculation of PAH activities was developed and validated. Mass spectrometry is a powerful technique with high sensitivity and specificity for quantification of compounds in complex biological matrices. The inclusion of stable isotopes of the analytes renders the method highly specific.

The LC-ESI-MSMS method was successfully applied for the direct quantification of Phe and Tyr in mouse brain tissue without PAH activity measurements. The tissue was extracted from wild type and PKU mice in order to assess diet effects on amino acids and neurotransmitters in the brain. The tissues were homogenized and directly prepared by the EZ:faast^{TM} kit and measured by MSMS. This constitutes a different application of the method described in Chapter 3. In this context, the method may be further extended by the quantification of Trp in mouse tissue. The only requirement for the extension of the method with other amino acids is the availability of stable isotopes of the corresponding amino acid.

During the setup of the *in vitro* expression system, we aimed at high expression levels for more accurate quantification of amino acids. However, as the MS method is far more sensitive and specific than the amino acid analyzer, the new method can also be used be with systems of lower transfection efficiencies and protein expression levels than presented in Chapter 3.

Many exonic *PAH* mutations investigated biochemically were studied before the awareness that up to 50% of missense mutations may influence splicing regulation (3, 4) and the action of the splicing code for exon recognition (5). The contribution of exon splicing enhancers and silencers, as well as of splice sites was poorly appreciated. But this influence may overrule what is predicted based on amino acid substitutions. In Chapter 4, the detailed analysis of *PAH* mutations in exon 11 has shown that splicing mutations masquerade as missense mutations and that exon 11 recognition is especially vulnerable. The combination of *in silico* analyses and *in vitro* assessment of mutations is highly important. Computer-based predictions provide helpful tools before embarking the genetic analysis on RNA level, but they also have to be considered critically. They may produce contradictory predictions on the effect of a mutation. This was observed for c.1139C>T mutation that was found causing exon 11 skipping by disrupting an ESE by ESEfinder 3.0. This was so far not confirmed in the patient cells and transfected minigenes.

All other exon 11 mutations reported up to date should be analyzed by the minigenes presented in Chapter 4. The production of several transcripts leads to reduced protein amounts and therefore to lower residual PAH activity than predicted. The c.1144T>C mutation for example and in general mutations causing reduced levels of wild type transcript may not respond in a 24-hour BH_4loading test. Low levels of protein with low residual activity may explain the delayed response. This will add to the consistency of genotype-phenotype correlations and possibly eliminate the need for a BH_4-loading test.

Unfortunately, we were not able to conclusively identify the splicing regulatory proteins binding exon 11 by RNA affinity chromatography to clarify the splicing mechanism of *PAH* exon 11. The sequence recognition is controlled by coordinate regulation of positive and negative proteins, which adds complexity to the identification of one single factor. We considered mass spectrometric analysis of the protein complexes and studying RNA interference. However, without a clear candidate regulatory protein mass spectrometry might lead to more confusion by identifying too many potential interaction partners. Further RNA affinity purifications are therefore planned in order to elucidate a splicing factor with the artificial mutations introduced in the minigene.

The new exon 11a detected upon overexpression of the splicing factors SF2/ASF and Srp40 in the minigenes will be further analyzed in a follow-up project. To our knowledge, it is the first report of a pseudoexon detected in *PAH*, even though it was generated under overexpression conditions of the splicing proteins. Eventually, the appearance of the pseudoexon could be triggered in the patient lymphoblasts by overexpression of the same

splicing regulatory proteins. Lymphoblast cell lines from PKU patients present a potent tool to study RNA processing (liver samples from patients are not available) and allow investigation of a mutation's effect at the genomic DNA level. Attention should be warranted to technical artifacts of RT-PCR and especially the quantification of aberrant versus normal *PAH* transcript is delicate with very low levels of expression. However, confirmatory experiments as described in Chapter 4 added important value to the molecular pathology of the assessed mutations in *PAH* exon 11. Finally, the BH_4 responsiveness of these missense mutations could be tested with the *in vitro* expression system outlined in Chapter 5.

The transient co-expression of two PAH subunits in a eukaryotic cell system provides rapid results for residual activity of the heteromeric PAH enzyme. The establishment of a system closely mimicking the patient genotype allows the investigation of sensitivity to BH_4 and elevated Phe levels. The analysis of numerous mutation combinations by the same system will enable the establishment of algorithms for the calculation of genotype-based BH_4 responsiveness. Chapter 5 describes the evaluation of only a few genotype combinations, but they contributed to highlight a few important aspects of such a system.

The comparison between two residual PAH activities must be controlled for variations in transfection efficiency, as seen for example by comparing absolute and normalized activities. The normalization of PAH activity with the activity of a co-transfected reporter plasmid is an advantageous method, as all parameters are measured in the same cell lysate. However, our method of choice, the β-galactosidase reporter assay, seems to be influenced by BH_4, leading to inconsistent results. Ma et al. (6) emphasized on the rigorous controlling of subunit contributions to heteromeric complexes and the analysis of subunit stoichiometry in co-transfection studies. An internal control consisting of quantitating a second mRNA produced by the same vector should be less susceptible to variation. An internal control is more favorable as upon co-transfection, the expression of both subunits in a significant population of cells is critical. It could be that a nonnegligible number of transfected cells express only a single PAH allele. An alternative to co-transfection of β-galactosidase reporter plasmid is the inclusion of a fluorescent protein-encoding transfection marker, which can be assessed by flow cytometry.

Some PAH mutants are more susceptible to accelerated degradation, and as could be seen from the immunoblotting of different PAH mutations in Chapter 3, protein amounts are variable depending on the type of a mutation. Thus, residual PAH activities need to be correlated with these differences in expression. Antibodies are available against total PAH (anti-PAH PH 8), but also for against FLAG- or Myc-tag fused to mutants. This allows the

quantification of total PAH and the amount of each mutant to be normalized with residual activity. It is not clear, whether transfecting different cDNA ratios would correct for differences in expression levels. A possibility for equalizing transcription levels between the two mutants would be the use of an inducible expression system. Commercial plasmid vectors are available containing for example the tetracycline inducible expression system for regulated control of transgene expression. Genes of interest can be fused to green or red fluorescent genes for expression monitoring and assessed by flow cytometry. Such a system would omit the need for performing another enzyme assay and provides values for expression levels of each plasmid vector.

The use of a bicistronic expression plasmid containing two *PAH* alleles, constitutes a further alternative approach. However, the introduction of two different *PAH* mutations in the same vector in the two *PAH* cDNA's requires the mutagenesis to be performed in a separate expression vector. The two complete *PAH* cDNA's then need to be replaced in every mutagenesis cycle in the final bicistronic expression vector.

If one identical *PAH* mutation is to be co-expressed in numerous combinations with other mutations, a few stable transfected cell lines with single mutations could be generated. This would ensure stable expression levels of one mutation and the second mutation would be transiently co-transfected. This approach absolutely requires the quantification of protein amounts and normalization of residual PAH activity for each PAH subunit.

As can be seen from above, the co-transfection of two subunits is a delicate approach with many parameters requiring adjustment. Advantageous for these mammalian expression systems is that proteins do not require purification and enzyme activities can be measured in the total protein extract. The *in vitro* experiments have shown that amounts of transiently expressed PAH proteins are high and stable enough for activity assay and immunoblotting.

Interesting would be also a co-immunoprecipitation of Myc- and FLAG-tagged PAH mutants co-expressed in COS-1 cells to illustrate an interaction between the two subunits after co-expression in COS-1 cells. A fraction of precipitated protein could then also be analyzed by mass spectrometry. Protein analysis by MS nowadays requires little amounts of protein material and is sensitive enough to distinguish small differences in mass. It could deliver information on the composition of the precipitated heteromeric PAH proteins.

PAH activity is tightly regulated by BH_4 and Phe. The established co-expression system with optimization of constant and identical expression levels allows testing various concentrations of both substrates. Although it is not a high-throughput system as described by Staudigl et al. (7), it is more specific, with lower detection limits and no interferences by

other fluorescing compounds. The *in vitro* PAH assay has also been adjusted for higher BH_4 levels to account for the optimal PAH working range, mentioned by Staudigl et al. (7).

In summary, a combination of standardized BH_4 loading test protocol, and the investigation of BH_4-response of known genotypes in a validated, eukaryotic cell system, is a highly powerful tool to clarify different aspects contributing to the diverse genotype-phenotype correlations encountered. The co-expression system accounts for dominance effects of one mutation to the phenotype, but also to BH_4-response, and deposit in a genotypes database like BIOPKU may greatly improve diagnosis and establish tailored treatment for the patient.

References

[1] Scriver, C. R. (2007). "The PAH gene, phenylketonuria, and a paradigm shift." Hum Mutat 28(9): 831-845.

[2] Pey, A. L., Stricher, F., et al. (2007). "Predicted effects of missense mutations on native-state stability account for phenotypic outcome in phenylketonuria, a paradigm of misfolding diseases." American journal of human genetics 81(5): 1006-1024.

[3] Lopez-Bigas, N., Audit, B., et al. (2005). "Are splicing mutations the most frequent cause of hereditary disease?" FEBS letters 579(9): 1900-1903.

[4] Wang, G. S. and Cooper, T. A. (2007). "Splicing in disease: disruption of the splicing code and the decoding machinery." Nature reviews. Genetics 8(10): 749-761.

[5] Barash, Y., Calarco, J. A., et al. (2010). "Deciphering the splicing code." Nature 465(7294): 53-59.

[6] Ma, Z. L., Werner, M., et al. (2007). "Quantitative analysis of cotransfection efficiencies in studies of ionotropic glutamate receptor complexes." Journal of neuroscience research 85(1): 99-115.

[7] Staudigl, M., Gersting, S. W., et al. (2011). "The interplay between genotype, metabolic state and cofactor treatment governs phenylalanine hydroxylase function and drug response." Human molecular genetics 20(13): 2628-2641.

Acknowledgments

I am sincerely grateful to Prof. Nenad Blau for the opportunity of this PhD thesis in the Division of Clinical Chemistry and Biochemistry in the Children's Hospital Zurich. Thank you very much for your supervision, your constant support, your confidence and advice, and the introduction to the field of inborn errors of metabolism. Thank you for the opportunity to learn many new techniques and enabling the research in Denmark.

I am grateful for the opportunity to collaborate with Prof. Brage S. Andresen in Odense, Denmark. Many thanks for inviting me to your lab for a great, valuable experience and thanks to your group members for their support. Thank you for introducing me to the interesting world of splicing and the research tools.

Thank you Prof. Beat Thöny, for your support, constructive inputs and advice, providing answers and scientific discussions. Thank you also for participating in my thesis committee and contributing to the guidance of this thesis.

I would like to thank Dr. Mübeccel Demirkol and Dr. Steven F. Dobrowolski for these fruitful collaborations and interesting projects.

Many thanks are expressed to Prof. François Verrey for accepting the guidance of this PhD thesis, as well as to Prof. Marc Donath for participating in my thesis committee.

I would like to thank Dr. Heinz Troxler for the good collaboration on the mass spectrometry project and his confidence in our project and myself for using the instruments. Many thanks go to present and former members of the MS group. And special thanks go to the Rita for help with samples and the Sciex2000, as well as to Daniela and Andreas for great technical assistance. In addition, I would like to thank Angela and Corinne for their help with the enzyme assay, and Anahita for HPLC measurements. Thanks also to the Clinical Chemistry Routine team for measuring the many samples at any time.

Thanks to all former and present members of the Clinical Chemistry and Metabolism division for contributing to the pleasant working atmosphere, always trying to help out and technical assistance.

I would like to thank the OP-U15 girls for the pleasant working atmosphere in the lab, helping out each other and the good times around. Thank you Sophia, Hiu Man, Imane, Dea, Vanessa, Magda, Adrienne, and Martin and to all the others in Clinical Chemistry, as well as in Metabolism and Hematology division for help and support, sharing ideas as well as enjoyable times at lunch and coffee breaks and outside the lab.

Many thanks go to my friends in and out of Zurich, for enjoyable times outside the lab, making these unforgettable. In the end I would like to send special thanks to my family for their encouragement, their support, advice, patience and love. And finally, I would like to sincerely thank Stefan for always encouraging and supporting me, for your patience and comprehension during these past years.

Thank you!

Curriculum Vitae

Caroline HEINTZ

Personal details

Born July 17, 1981 in Luxembourg
Nationality: Luxembourgish
caroline.heintz@alumni.ethz.ch

Education

04/2008 – 06/2012	**PhD Thesis, University Children's Hospital Zurich** "MOLECULAR AND METABOLIC BASES OF TETRAHYDROBIOPTERIN-RESPONSIVE PHENYLALANINE HYDROXYLASE DEFICIENCY" Division of Clinical Chemistry and Biochemistry Supervision by Prof. Nenad Blau, University of Zurich
10/2007	**Master of Science ETH in Chemistry, ETH Zurich**
04/2006 – 07/2006	**Master Thesis** "DIRECTED EVOLUTION OF A MOLTEN GLOBULAR ENZYME" Prof. Donald Hilvert, ETH Zurich
08/2006 – 09/2006	**Research Project II** "SYNTHESE VON NEUEN B-SEKRETASE INHIBITOREN – DIE SUCHE NACH WIRKSAMEN ALZHEIMER MEDIKAMENTEN" Prof. François Diederich, ETH Zurich
04/2006	**Bachelor of Science in Chemistry, ETH Zurich**
04/2005 – 07/2005	**Research Project I** "*IN VITRO* CHARACTERIZATION OF IMPROVED *E.COLI* CHORISMATE MUTASE HINGE-LOOP VARIANTS" Prof. Donald Hilvert, ETH Zurich
10/2001	**Chemistry studies** ETH Zurich, Switzerland
09/1993 – 07/2001	**Diplôme de fin d'études secondaires** Major in natural sciences (Type C) Lycée Michel Rodange Luxemboug

Supervising Experience

09/2008 – 12/2010	Assistance in practical courses in physiology for biology and medical students Department of Physiology, University of Zurich
01/2008 – 02/2008	Supervising assistant for general chemistry laboratory course students Department of Chemistry and Applied Biosciences, ETH Zurich

International

01/2010 – 03/2010	Visiting Scientist, University of South Denmark Prof. Brage Andresen
05/2007 – 06/2007	Internship - Scientific assistant to the research project "Molecular mechanisms leading to the inhibition of erythroid differentiation by mediators of inflammation" at 'Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Luxembourg' Supervisor: Dr. Marc Diederich
09/1998 – 07/1999	Student exchange in USA High School stay in Augusta, Georgia

Publications (prior to the PhD)

1) Neuenschwander, M.; Butz, M.; Heintz C.; Kast, P.; Hilvert D.; A simple selection strategy for evolving highly efficient enzymes. *Nature Biotechnology* 2007, 25, (10), 1145 – 1147.

2) Buck, I.; Morceau, F.; Christofanon, S.; Heintz, C.; Chateauvieux, S.; Reuter, S.; Dicato, M.; Diederich, M. Tumor necrosis factor alpha inhibits erythroid differentiation in human erythropoietin-dependent cells involving p38 MAPK pathway, GATA-1 and FOG-1 downregulation and GATA-2 upregulation. *Biochemical Pharmacology* 2008, 76, (10), 1229 – 1239.

Oral and Poster Presentations

11th International Congress of Inborn Errors of Metabolism (ICIEM), San Diego, 2009

Oral	Genotypes and BH_4 responsiveness in 600 Turkish PKU patients
Poster	Genotype-predicted BH_4 responsiveness and molecular genetics in Croatian patients with phenylalanine hydroxylase (PAH) deficiency

University Children's Hospital, Zurich, 2009

Poster	Genotype-predicted tetrahydrobiopterin (BH_4) responsiveness and molecular genetics in Croatian patients with phenylalanine hydroxylase (PAH) deficiency

42nd European Metabolic Group (EMG) Meeting, Lisbon, 2010

Oral	Genotype-based prediction of BH_4 responsiveness in phenylketonuria

6th Zurich Center for Integrative Human Physiology (ZIHP) Symposium, Zurich, 2010

Poster	Molecular pathology of mutations in *PAH* exon 11, impact on mRNA processing, and potential impact on therapy with 6R-tetrahydrobiopterin (BH_4)

Society for Study of Inborn Errors of Metabolism (SSIEM) Annual Symposium, Istanbul, 2010

Oral	Molecular pathology of mutations in *PAH* exon 11, impact on mRNA processing, and potential impact on therapy with 6R-tetrahydrobiopterin (BH_4)
Poster	Genotype-based prediction of BH_4-responsiveness in PKU

Society for Study of Inborn Errors of Metabolism (SSIEM) Annual Symposium, Geneva, 2011

Oral	Quantification of PAH activity in cells and tissues using stable isotope dilution LC-MS/MS

Tri-National Congress of Swiss Society of Clinical Chemistry (SSCC), Zürich, 2011

Poster	Novel Assay for Quantification of Phenylalanine Hydroxylase Activity by Isotope-Dilution LC-MS/MS

1st Forschungszentrum für das Kind (FZK) Retreat, Halbinsel Au, Zürich, 2011

Oral	Molecular pathology of mutations in *PAH* exon 11, impact on mRNA processing, and potential impact on therapy with 6R-tetrahydrobiopterin (BH_4)

4th European PKU Group Meeting (EPG), Rome, 2012

Oral	Molecular characterization and genotype/phenotype correlations in BH_4-responsive PKU